Identification of a Novel Polymorphism in the 3’UTR of the L-Arginine Transporter Gene SLC7A1
Contribution to Hypertension and Endothelial Dysfunction

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Background—Endothelial dysfunction because of reduced nitric oxide bioavailability is a key feature of essential hypertension. We have found that normotensive siblings of subjects with essential hypertension have impaired endothelial function accompanied by altered arginine metabolism.

Methods and Results—We have identified a novel C/T polymorphism in the 3’UTR of the principal arginine transporter, solute carrier family 7 (cationic amino acid transporter, y+ system), member 1 gene (SLC7A1). The minor T allele significantly attenuates reporter gene expression (P<0.01) and is impaired in its capacity to form DNA-protein complexes (P<0.05). In 278 hypertensive subjects the frequency of the T allele was 13.3% compared with 7.6% in 498 normotensive subjects (P<0.001). Moreover, the overall genotype distribution observed in hypertensives differed significantly from that in normotensives (P<0.001). To complement these studies, we generated an endothelial-specific transgenic mouse overexpressing L-arginine transporter SLC7A1. The Slc7A1 transgenic mice exhibited significantly enhanced responses to the endothelium-dependent vasodilator acetylcholine (−log EC50 for wild-type versus Slc7A1 transgenic: 6.87±0.10 versus 7.56±0.13; P<0.001). This was accompanied by elevated production of nitric oxide by isolated aortic endothelial cells.

Conclusions—The present study identifies a key, functionally active polymorphism in the 3’UTR of SLC7A1. As such, this polymorphism may account for the apparent link between altered endothelial function, L-arginine, and nitric oxide metabolism and predisposition to essential hypertension. (Circulation. 2007;115:1269-1274.)

Key Words: amino acids • endothelium • genes • genetics • hypertension • molecular biology • nitric oxide
NM_003045 for mRNA, NT_009799 for genomic sequence) were used as background and positive control, respectively, for luciferase assays. Human DNA with homologous polymorphisms (any CT or TT) was used as a positive template to generate allele-specific amplicons. Primer sets p047-p049 and p050-p050 were again used to generate 216-bp PCR products containing allele C and allele T, respectively. Other primers were also used to generate different sized allele-specific amplicons as well as “nonrelated” DNA from the last intron of SLCA7A1. All DNA fragments were digested downstream of luciferase gene at an XbaI site, immediately preceding the poly(A) sequence. pSV-β-Galactosidase control vector (Promega) was used as internal control to correct for transfection efficiency among samples.

Chinese hamster ovary cells were grown at 37°C under 5% CO2 in DMEM ( Gibco/BRL) supplemented with 2 mmol/L l-glutamine and 10% heat-inactivated FCS. At approximately 70% confluence, the cells were cotransfected with equimolar amounts of each reporter gene construct and 2 μg internal control DNA (pSV-β-Galactosidase control vector) by electroporation in 0.45-cm cuvettes. The electroporation conditions were 500 μF, 270 V. After incubation for 24 and 48 hours after transfection, cells were washed twice with PBS, harvested in the reporter lysis buffer provided in the luciferase assay system (Promega), and centrifuged at 13 000 rpm for 1 minute at 4°C. The supernatant was assayed for both luciferase and β-galactosidase activities. β-Galactosidase activity was measured colorimetrically with the use of Emax precision microplate reader (Molecular Devices, Sunnyvale, Calif). Luciferase activity was normalized to β-galactosidase activity to correct for differences in transfection efficiency. All of the assays were performed in triplicate, and the means of relative luciferase activity were plotted as percent with respect to pGL3-TK. At least 3 independent experiments were performed for each reporter gene construct.

**Generation and Genotyping of Transgenic Mice Overexpressing SLC7A1-GFP**

To establish the functional impact of an alteration in endothelial SLC7A1 expression in the range of that predicted by the reporter studies, we established an endothelial-specific Slc7A1 overexpressing transgenic mouse. Plasmid pT2BLacZpA1L7 containing mouse TIE2 promoter and longer enhancer fragment was a gift from Dr Thomas N. Sato (University of Texas Southwestern Medical Center at Dallas). mCAT-1-GFP was cloned into EcoRI-BamHI sites, followed by the insertion of TIE2 promoter fragment into the HindIII site of pBluescript KS(−) (Invitrogen, Carlsbad, Calif), resulting in the generation of plasmid pBSTIE2CAT-1gfp. The TIE2 longer enhancer fragment (~10.6 kb), released from the plasmid pT2BLacZpA1L7 by complete digestion with NotI and partial digestion with XhoI, was then inserted into pBSTIE2CAT-1gfp. All the cloning products, as well as the mCAT-1-GFP fusion, were confirmed by enzyme digestion and sequencing.

The resulting plasmid, containing Slc7a1-GFP driven by TIE2 promoter and enhancer, was digested by SalI to remove the vector backbone. The remaining gene expression cassette was then gel-purified, resuspended in injection buffer (10 mmol/L Tris-HCl, pH 7.4, 0.1 mmol/L EDTA), and passed through a 0.45-μm filter (Millipore, Billerica, Mass) before microinjection into oocytes from C57Bl/6 mice. To screen for positive transgenic mice, 4 primer sets, each with at least 1 primer binding to GFP sequences, were used to amplify DNA and mRNA from mouse samples. PCR using the 3 primer pairs, 5'-CTTGTGTCAGGCCGGGTAC-3' (pri502) and 5'-CTGACAGCACTTTGGACCGACG-3' (pri523), 5'-GTCCTCCTTGTGTTGACATCG-3' (pri524); 5'-TCGTTGACACCCACCTGCTTTAC-3' (pri532), 5'-GATGTCGGTGTGCGGCAGTCTC-3' (pri530); and 5'-GAGCAGGACGAAGCTCTTACTTT-3' (pri531), was performed by denaturation at 94°C for 3 minutes, followed by 35 cycles of 90°C for 30 seconds, 65°C for 30 seconds and 72°C for 2 minutes, and a final extension step at 72°C for 10 minutes. PCR involving primers 5'-CTGATGGAGCCTCTATGACTGATGGATGATG-3' (pri509) and 5'-AAAGGCACCTGCGGCAAGCTG-3' (pri508) was performed under similar conditions except that the annealing temperature was 60°C and the extension time at each cycle was 3.5 minutes. Transgenic-positive mice were maintained by backcrossing to wild-type C57Bl/6 and subjected to further PCR screening of their offspring.

**Isolation and Fluorescence-Activated Cell Sorting of Endothelial Cells From Mouse Aorta**

Primary murine aortic endothelial cells were isolated from both wild-type and transgene-positive C57Bl/6 mice (Baker Institute Animal Center, Melbourne, Australia) as described. Briefly, aortas were harvested, the adventitia was removed, and strips were plated lumen side down into Matrigel in culture medium ECM (each 500 mL containing 200 mL of DMEM without FCS, 200 mL of Ham’s F12 media (Invitrogen), 100 mL of FCS, 15 mg of endothelial mitogen, 30 mg of heparin, and 1 mL of antibiotic/antimycotic). The isolated murine aortic endothelial cells were further purified by fluorescence-
ence-activated cell sorting (FACS) (model FACSaria, Becton Dickinson, Franklin Lakes, NJ) by utilizing their property of uptake of acetylated low-density lipoprotein (Ac-LDL). Briefly, murine aortic endothelial cells were incubated with DMEM containing 2 μg/mL Dil-Ac-LDL for 4 to 16 hours, were washed 3 times with PBS, and then were resuspended in FACS sorting buffer (each 100 mL S-MEM-Ca2+/free medium containing 2 mL of 0.5 mol/L EDTA, 2 mL of antibiotic/antimycotic, 1 mL of FCS). A 418-nm laser was used for excitation and 550 nm for emission, “scatter gates” were set to minimize the contribution of cell pairs, and “fluorescence gates” were chosen to eliminate the more highly fluorescent macrophages. The sorted cells were termed SLC7A1–murine aortic endothelial cells. They were maintained for up to 8 passages and used for experiments from passages 3 to 6.

Endothelial Function in Mouse Aortic Rings
Aortic rings were prepared from wild-type and Slc7a1 transgenic mice (10 to 14 weeks old) as described previously. In brief, aortic ring segments (2 mm in length) were mounted into an isometric myograph (myograph model 610 mol/L, JP Trading, Copenhagen, Denmark). After a 30-minute equilibration period, each chamber was subjected to a passive length-tension stretch. This procedure enabled each vessel to be normalized to an internal circumference equivalent to 90% of the transmural pressure of 100 mm Hg. Endothelial integrity was determined initially by the demonstration of at least 50% vasodilation to 1 μmol/L acetylcholine. Full concentration-response curves to acetylcholine (1 nmol/L to 100 μmol/L) were constructed with the use of vessels preconstricted with cicazoline. At a concentration that achieved 70% of the contraction induced by KPSS (in mmol/L: KCl 124, KM2PO4 1.18, MgSO4 1.17, NaHCO3 25, CaCl2 2.5, EDTA 0.026, glucose 5.5 at pH 7.4).

Endothelial Arginine Transport and NO Production
To examine the effect of endothelial Slc7a1 transgene expression, we compared the cellular uptake of [3H]-arginine in wild-type and Slc7a1 transgenic aortic endothelial cells, isolated as above. [3H]-Arginine was measured as described previously. In conjunction, the influence of SLC7A1 overexpression on NO production was determined with the NO fluorochrome 4-amino-5-methylamino-2′,7′-dichlorofluorescein diacetate (DAF-FM, Molecular Probes, Eugene, Ore), as described previously.

Statistical Methods
Data are presented as mean ± SEM. Between-group comparisons were performed with use of unpaired Student t tests for normally distributed data or χ2 tests for categorical data. The authors had full access to and take full responsibility for the integrity of the data. All authors read and agree to the manuscript as written.

Results
To discover potentially relevant SNPs in the exons of SLC7A1, we sequenced all 12 exons using pooled and individual DNA samples after amplification of each using individual DNA samples after amplification of each using flanking PCR primers. This identified a novel SNP located at nucleotide 2178 in the SLC7A1 3′ UTR, 10 nucleotides (nt) downstream of the stop codon (nm_003045). Details of this SNP, subsequently referred to as ss52051869, have been placed online (www.ncbi.nlm.nih.gov/projects/SNP/snp_ss.cgi?ss=52051869).

To evaluate whether ss52051869 differentially affects SLC7A1 expression, we tested the effect of 3′ UTR segments of contrasting genotype (CC versus TT) on luciferase reporter expression. Irrespective of genotype, the 3′ UTR segment reduced luciferase expression. Notably, however, cells transfected with C allele construct (pGL3-TK-216-bp-Allele-C) had significantly higher luciferase activity than cells transfectected with T allele construct (pGL3-TK-216-bp-Allele-T) (Figure 1). Constructs made with “nonrelated” DNA of similar size showed no alteration in reporter expression (data not shown). Furthermore, no inhibitory effects were seen when smaller DNA fragments were used, for example, a 114-bp DNA insert containing the polymorphism site (representing nt 2161 to 2174 of nm_003045), in the reporter gene assay (data not shown).

We performed gel shift assays to investigate whether differences in allelic expression between C and T allele variants could be attributed to the differential binding of nuclear proteins. In these assays, 2 PCR amplicons corresponding to the sequence from nt 2161 to 2376 in the 3′ UTR were 32P-labeled and allowed to interact with HeLa nuclear extracts. Both probes formed DNA-protein complexes and showed a similar pattern of migration on the gel. The allele C fragment, however, demonstrated much stronger binding than allele T (P<0.05) (Figure 2). To determine the binding specificity of these DNA-protein complexes, competition experiments were performed with 100-fold excess of unlabeled probes before the addition of nuclear extracts. DNA-protein complex formation was completely abrogated by unlabeled probe (Figure 2). To determine the identity of the nuclear proteins that bound, we performed additional competition electrophoretic mobility shift assays using the consensus oligonucleotides containing AP1, AP2, CREB, nuclear factor-κB, OCT1, SP1, and TFIIH binding elements. In particular, the preincubation of consensus oligonucleotides containing SP1, AP1, AP2, CREB, and TFIIH binding elements resulted in abolition of the DNA-protein complexes, consistent with them being specific competitors (data not shown).

Given the role of the 3′ UTR in the regulation of mRNA expression, we determined whether there was a difference in ss52051869 allele frequency between hypertensive and normotensive subjects. In hypertensive subjects the frequency of the T allele was 13.3% compared with 7.6% in the normo-
tensive subjects \((P<0.001)\). Moreover, the genotype distribution observed in hypertensives differed significantly from that in normotensives, as shown in the Table. With regard to the \(TT\) genotype specifically, this was observed in 1 normotensive subject (0.2%) and 6 hypertensive subjects (2.2%). All genotype frequencies accorded with Hardy-Weinberg equilibrium.

To evaluate the in vivo effect of altered \(SLC7A1\) expression, we established endothelial-specific overexpression in a transgenic mouse. Full kinetic analysis of arginine transport in aortic endothelial cells from wild-type and \(Slc7a1\) transgenic mice showed a significant increase in \(V_{\text{max}}\) (2149 ± 24 versus 1513 ± 39 pmol/mg per minute, respectively; \(P=0.045\)), consistent with increased \(SLC7A1\) expression (Figure 3A). Of note, the magnitude of the difference in arginine transport capacity in endothelial cells from wild-type and transgenic mice was in the range of that which might be expected for the effect of the major and minor SNP alleles on the basis of the reporter studies. In conjunction with the elevation of arginine transport, aortic endothelial cells from \(Slc7a1\) transgenic mice also demonstrated a highly significant increase in NO production (Figure 3B).

Having demonstrated the cellular effect of modest \(SLC7A1\) overexpression, we next investigated the effect on vascular pharmacology in isolated aortic rings. Vascular rings obtained from \(Slc7a1\) transgenic mice showed significantly greater sensitivity (\(EC_{50}\)) to the endothelium-dependent vasodilator acetylcholine (Figure 4), whereas responses to the endothelium-independent vasodilator sodium nitroprusside were not altered (data not shown).

**Discussion**

Intracellular L-arginine is derived predominantly from the extracellular milieu and transported principally via the type 1

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**Table 1:** Genotype and Allele Frequencies of 3' UTR Polymorphism of \(SLC7A1\) in Hypertensive and Normotensive Subjects

<table>
<thead>
<tr>
<th>Group</th>
<th>N</th>
<th>CC</th>
<th>CT</th>
<th>TT</th>
<th>(\chi^2)</th>
<th>(P)</th>
<th>C</th>
<th>T</th>
<th>(\chi^2)</th>
<th>(P)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normotensive</td>
<td>498</td>
<td>423 (84.9%)</td>
<td>74 (14.9%)</td>
<td>1 (0.2%)</td>
<td>15.2</td>
<td>&lt;0.001</td>
<td>920 (82.4%)</td>
<td>76 (7.6%)</td>
<td>11.6</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Hypertensive</td>
<td>278</td>
<td>210 (75.5%)</td>
<td>62 (22.3%)</td>
<td>6 (2.2%)</td>
<td></td>
<td></td>
<td>482 (86.7%)</td>
<td>74 (13.3%)</td>
<td></td>
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</tbody>
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**Figure 2.** A, Electrophoretic mobility shift assays for 3'UTR SNP of \(SLC7A1\). \(^{32}\)P-labeled 3'UTR DNA fragments containing different alleles of the polymorphism (probes “allele C” or “allele T”) were mixed with or without HeLa nuclear extracts to investigate whether there was specific nucleotide–protein binding. Lane 1, allele C probe; lane 2, allele C probe plus HeLa extract; lane 3, excess unlabeled allele C DNA plus HeLa extract plus allele C probe; lane 4, allele T probe; lane 5, allele T probe plus HeLa extract; lane 6, excess unlabeled allele T DNA plus HeLa extract plus allele T probe. B, Quantification of electrophoretic mobility shift assay results for 3'UTR SNP of \(SLC7A1\). Both allele C and allele T probes were able to specifically bind to nuclear extracts in an allele-specific manner, with the allele C probe exhibiting much greater binding than that for allele T \((P=0.0069)\). Group data (mean ± SEM) are obtained from 3 separate experiments.

**Figure 3.** A, L-Arginine transport kinetic curves in aortic endothelial cells from \(Slc7a1\) transgenic (open circles) and wild-type (closed circles) mice. \(^{#}\)\(P=0.045\). B, NO production (as measured by 4-amino-5-methylamino-2',7'-dichlorofluorescein [DAF] fluorescence) by endothelial cells from \(Slc7a1\) transgenic and wild-type mice. \(^{*}\)\(P<0.001\). RU indicates relative units.
cationic amino acid transporter SLC7A1.7 Clinical and experimental paradigms involving an extracellular deficiency of l-arginine or its transport have been shown to be associated with reduced endothelial function and NO production.11–13 Furthermore, the administration of l-arginine to hypertensive animals and humans has been shown to reduce blood pressure and to restore endothelial function in both hypertensive subjects and those with a genetic predisposition toward hypertension.8,14 The present study has found that a functional variant of the l-arginine transporter gene SLC7A1 is increased in frequency in subjects with essential hypertension and that, in experimental models, altered expression of SLC7A1 results in physiologically relevant changes in NO production and endothelial function.

Although hypertension demonstrates significant familial aggregation, genomewide linkage studies have not provided consistent results, and in general the strength of various associations between putative loci of interest and blood pressure has been modest.15 At the same time, strong evidence supports the notion of environmental inputs into the subsequent development of hypertension, including overweight and elevated salt intake.16 In this context, acute intervention studies indicate that l-arginine may influence blood pressure.17 More generally, recent dietary intervention studies raise the possibility that combination diets rich in vegetables, nuts, and grains could exert influences on blood pressure by mechanisms beyond the effects of sodium reduction alone. In particular, the Dietary Approaches to Stop Hypertension (DASH) diet was shown to exert antihypertensive effects independent of sodium.18 As such, it has been shown that nuts alone improve endothelial function, and this may be accounted for by their significant l-arginine content.19

To establish the functional importance of relatively modest changes in SLC7A1 expression, we established an endothelial-specific transgenic mouse. Endothelial cells obtained from transgenic mice displayed a marked increase in NO under basal conditions, and isolated aortic rings from these mice showed evidence of increased endothelial function. It is acknowledged that in the generation of the Slc7a1 transgenic mice we did not specifically demonstrate the effect that might be observed in the context of restoration of l-arginine transport in a clinical paradigm or by a “knock-in” mouse model of reduced l-arginine transport. Nevertheless, these experimental findings are directionally consistent with the expected effects of increased substrate availability for NO synthesis. In conjunction with these data, we showed recently that another well-recognized cardiovascular risk factor, cigarette smoking, significantly reduced l-arginine transport and NO production in endothelial cells.20 Taken together, the present study and our previous work strongly support the notion that genetic or environmental alterations in l-arginine transport have the capacity to directly influence NO production and thereby vascular tone.

SLC7A1 is a high-affinity, low-capacity cationic amino acid transporter that facilitates uptake of arginine and lysine in mammalian cells. SLC7A1 is expressed almost ubiquitously, with the exception of adult liver, but its expression level varies considerably in different tissues and cell types.20 SLC7A1 expression can be modulated by a variety of stimuli including cell proliferation, growth factors, cytokines, certain hormones, microRNA, nutrients, and cellular stress, including amino acid deprivation.20 Interestingly, the 3’UTR of human SLC7A1 mRNA contains several potential target sites for miR-122, a liver-specific microRNA. Indeed, it was shown recently that activity of the endogenous SLC7A1 mRNA was translationally repressed by miR-122, and such repression could be reversed by the binding of HuR, an AU-rich element binding protein, to the 3’UTR of SLC7A1 mRNA.21 Our findings on the SNP in the 3’UTR of SLC7A1 mRNA are therefore consistent with the important regulatory role that the 3’UTR plays in controlling gene expression. In the present study, however, we did not directly correlate the genotype with SLC7A1 mRNA or protein expression because of the inability to obtain relevant vascular tissue or cells for such investigations.

In conclusion, we have identified a functionally relevant SNP in the 3’UTR region of SLC7A1, the principal l-arginine transporter in humans. In the context of hypertension, this finding provides the basis for an interaction between a genetically programmed influence on vascular endothelial function and blood pressure with environmental factors, including diet and traditional cardiovascular risk factors.

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Disclosures
None.

References
Hypertension currently affects ≈25% of the population in westernized societies. Despite the high prevalence of hypertension and the long history of hypertension research, the pathogenesis of essential hypertension remains controversial. Most current theories suggest that essential hypertension results from a complex set of gene-environment interactions. One hallmark of hypertension is the presence of abnormal endothelial function. Interestingly, this phenomenon is commonly also observed in nonhypertensive siblings of individuals with hypertension. In the present study, we report, in hypertensive subjects, the increased presence of a novel polymorphism in a key gene responsible for the delivery of arginine into cells. We demonstrate that its presence can alter endothelial function and nitric oxide production, thereby indicating its potential role in the pathogenesis of hypertension. Identification of this gene polymorphism in hypertensives may in the future assist in the selection of certain antihypertensive interventions.
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