Reduction of Gstm1 Expression in the Stroke-Prone Spontaneously Hypertension Rat Contributes to Increased Oxidative Stress


Abstract—Human essential hypertension is a classic example of a complex, multifactorial, polygenic disease with a substantial genetic influence in which the underlying genetic components remain unknown. The stroke-prone spontaneously hypertensive rat (SHRSP) is a well-characterized experimental model for essential hypertension and endothelial dysfunction. Previous work, identified glutathione S-transferase μ type 1, a protein involved in detoxification of reactive oxygen species, as a positional and functional candidate gene. Quantitative real-time polymerase chain reaction showed a highly significant, 4-fold reduction of glutathione S-transferase μ type 1 mRNA expression in 5- and 16-week-old SHRSP compared with the congenic and normotensive Wistar Kyoto rats. This suggests that differential expression is not attributable to long-term changes in blood pressure. DNA sequencing identified one coding single nucleotide polymorphism (R202H) and multiple single nucleotide polymorphisms in the promoter region. mRNA expression changes were reflected at the protein level, with significant reductions in the SHRSP glutathione S-transferase μ type 1. Protein was colocalized with aquaporin 2 to the principle cells of the renal collecting ducts. Coupled to significant increases in nitrotyrosine levels in the kidney, this suggests a pathophysiological role of this protein in hypertension and oxidative stress. Similar processes may underlie oxidative stress in the vasculature.

Key Words: rats, stroke-prone SHR ■ hypertension, genetic ■ gene expression ■ oxidative stress

H uman essential hypertension is a classic example of a complex, multifactorial, polygenic disease with a substantial genetic influence in which the underlying genetic components remain unknown.1–4 The methodological difficulties in studying the genetic determinants of hypertension have given a major impetus for development of similar but inherently simpler paradigms in rodent models of genetic hypertension that remain under complex control.5–7 Genetic heterogeneity can be reduced by the use of inbred strains with complete control over environmental influences. Moreover, the ability to produce genetic crosses and analyze large numbers of progeny facilitate genetic analysis, including genetic dissection of complex phenotypes, gene–gene and gene–environment interactions.8–10 The stroke-prone spontaneously hypertensive rat (SHRSP) is a well-characterized experimental model for essential hypertension and endothelial dysfunction.11–13 Genome-wide scans performed on several rat crosses have identified quantitative trait loci (QTLs), which are involved in blood pressure regulation.5 However, these represent large chromosomal regions and contain too many putative candidate genes to pursue classic positional cloning strategies. More recently, generation of congenic strains in which blood pressure QTLs from a normotensive strain have been introgressed into a hypertensive background has allowed genetic dissection of QTLs in hypertensive strains.13–16 Recent studies using a combination of the SP.WKYGla2c* congenic strain, derived from SHRSPGla and WKYGla, and genome-wide microarray expression profiling, have shown significantly reduced expression of glutathione S-transferase μ type 1 (Gstm1) in kidneys of SHRSPGla compared with SP.WKYGla2c* congenic and WKYGla controls.17 Gstm1 forms a superfamily, the dimeric proteins of which catalyze a number of distinct glutathione-dependent reactions but can also function as peroxidases and isomerases.18–20 The primary role of Gstm1 is the metabolism of a broad range of reactive oxygen species (ROS) and xenobiotics, and they have been implicated in a number of diseases, including childhood asthma21 and lung cancer,22 although little work has been undertaken in cardiovascular disease and hypertension.
The current study was designed to investigate renal expression and cellular localization of GSTM1 together with parallel measurements of oxidative stress in renal and vascular tissues.

**Methods**

Rat Strains

Inbred colonies of SHRSP<sub>Gl</sub> and WKY<sub>Gl</sub> have been developed at the University of Glasgow since 1991, as described previously. The congenic strain used in the current study contains a 22-cM segment transferred from WKY<sub>Gl</sub> to the genetic background of SHRSP<sub>Gl</sub> using a marker-assisted “speed” congenic strategy as described previously. The full name of this strain is SP.WKY<sub>Gl</sub>2 (D2Wox9-D2 Mgh12), abbreviated to SP WKY<sub>Gl</sub>2<sup>c</sup> for simplicity. Brown Norway (BN) and the spontaneously hypertensive rat (SHR) strains were purchased from Harlan. These studies were approved by the home office according to regulations regarding experiments with animals in the United Kingdom, which are equivalent to US regulations.

Quantitative Real-Time Polymerase Chain Reaction

Renal total RNA was extracted from 5- and 16-week-old rats using RNeasy kits (Qiagen), treated with DNase Free (Ambion), and accurately quantified using Ribogreen (Molecular Probes). Normalization was confirmed by performing real-time polymerase chain reaction (PCR) on either Lightcycler (Roche) or TaqMan (Applied Biosystems) of β-actin (Promega) with comparable threshold cycle values. Lightcycler RT-PCR was performed in a single run with 0.5 μmol/L each primer for Gstm1 F5'-TTT GAG CCC AAG TGC CTG GA-3' and R 5'-GCA GGA TCC AAT GTG GAC AG-3'. A standard curve was generated from various dilutions of cloned, linearized Gstm1 plasmid. TaqMan probes for Gstm1 (Rn00755117m1-labeled FAM) and β-actin (4352340E-labeled VIC) were multiplexed. Expression of Gstm1 relative to β-actin in each sample was derived using the comparative (∆∆CT) method.

Sequencing

Two strategies were used to sequence the Gstm1 gene from SHRSP<sub>Gl</sub>, SP.WKY<sub>Gl</sub>2<sup>c</sup>, WKY<sub>Gl</sub>, and the SHR and BN. Renal RNA was used as a template for RT-PCR amplifying Gstm1 from each of the 5 strains. PCR primers were also designed to exon 1 and genomic DNA regions upstream of the Gstm1 using BN genome sequence as a template (supplemental Table I, available online at http://www.hypertensionaha.org). Gstm1 upstream sequences from rat Gstm1, mouse gstm1 and gstm3, and all human GSTM family were obtained from ENSEMBL and compared using rVISTA 2.0, and putative promoter regions were inferred from the transcriptional database.

Western Analysis of GSTM1 and Nitrotyrosine in Rat Kidney

Kidneys were removed rapidly from terminally anesthetized 16-week-old SHRSP<sub>Gl</sub>, SP.WKY<sub>Gl</sub>2<sup>c</sup>, WKY<sub>Gl</sub>, and the SHR and BN. Renal RNA was used as a template for RT-PCR amplifying Gstm1 from each of the 5 strains. PCR primers were also designed to exon 1 and genomic DNA regions upstream of the Gstm1 using BN genome sequence as a template (supplemental Table I, available online at http://www.hypertensionaha.org). Gstm1 upstream sequences from rat Gstm1, mouse gstm1 and gstm3, and all human GSTM family were obtained from ENSEMBL and compared using rVISTA 2.0, and putative promoter regions were inferred from the transcriptional database.

Immunohistochemistry on Rat Kidneys

Wax sections (5 μm) were obtained from kidneys of 16-week-old SHRSP<sub>Gl</sub> and WKY<sub>Gl</sub> male rats. After treatment with antigen retrieval solution (DAKO), sections were blocked with 20% serum in PBS for 1 hour. Antibodies were incubated overnight at 4°C with an antibody against GSTM1 and then aquaporin 2 (AQP2). Washing with PBS was followed by incubation with a mixture of secondary antibodies, anti-rabbit IgG–fluorescein isothiocyanate and donkey anti-goat IgG conjugated with Alexa Fluor 350 (Molecular Probes), at room temperature for 1 hour. Fluorescence was detected on the Olympus BX40 fluorescent microscope using the U-MWIB and U-MWU cubes.

Superoxide Levels in Kidney and Vascular Tissues

Superoxide was measured using 20 μmol/L lucigenin chemiluminescence in thoracic aorta as described previously. Similar methods were used for kidney tissue dissected into renal medulla and cortex. The tissue was homogenized in 0.05 mol/L phosphate buffer, pH 7.8, and detected with 5 μmol/L lucigenin. Frozen sections of thoracic aorta were prepared (30 μm). Samples were incubated with hydroethidine (2 μmol/L for 20 minutes) and fluorescence detected using a Bio-Rad laser scanning confocal microscope. SHRSP<sub>Gl</sub>, WKY<sub>Gl</sub>, and SP.WKY<sub>Gl</sub>2<sup>c</sup> samples were analyzed in parallel. Dedicated software was used to measure average fluorescence of each vessel.

Results

Lightcycler Quantitative Real-Time PCR

Renal Gstm1 mRNA levels were assessed by quantitative real-time PCR (qRT-PCR) in male rats at 5 weeks of age, before the onset of significant hypertension in the SHRSP<sub>Gl</sub>, and at 16 weeks of age, when hypertension is fully established (Figure 1). There were significant reductions in Gstm1 mRNA in the SHRSP<sub>Gl</sub> compared with SP.WKY<sub>Gl</sub>2<sup>c</sup> and WKY<sub>Gl</sub> in 5- and 16-week age groups with similar levels in SP.WKY<sub>Gl</sub>2<sup>c</sup> and Wistar Kyoto rats (WKY).

Sequencing of Rat Gstm1

Sequencing of SHRSP<sub>Gl</sub>, SHR, SP.WKY<sub>Gl</sub>2<sup>c</sup>, and WKY<sub>Gl</sub> Gstm1 exons identified a nonsynonymous single nucleotide polymorphism (SNP), changing arginine 202 (Arg 202) to...
histidine (R202H) in the SP.WKY\textsubscript{GLA}2c\textsuperscript{*} congenic and WKY-\textsubscript{GLA} strains. The same amino acid was identified in the BN rat but not in other \textit{Gstm} isoforms from rat, mouse, and human (Figure 2A; Table). In addition, the crystal structure of rat \textit{GSTM1}\textsubscript{27} was referred to model the potential effects of the substitution of R202H, in which Arg 202 was found to be surface located and distal from the active site (data not shown). Using genomic DNA as template, sequencing was extended to include the upstream, regulatory regions and identified 11 SNPs, an insertion, and a deletion (Figure 2A). In all cases, sequence differences between the SHRSP\textsubscript{GLA} and SHR were also found between SHRSP\textsubscript{GLA} and BN (Figure 2A). Moreover, these sequence differences were predictive for renal \textit{Gstm1} expression levels in each of the strains (Figure 2B). rVISTA 2.0 plots identified conserved sequences in the putative promoter between rat \textit{Gstm1}, mouse \textit{gstm1}, mouse \textit{gstm3}, and human \textit{GSTM1} and \textit{GSTM5}. Of the 11 SNPs identified, 3 were localized to conserved transcription factor binding sites: acute myloid leukemia (AML1) and hepatic nuclear factor 4 sites (HNF4).

**Western Analysis**

To determine whether the significant reduction in \textit{Gstm1} mRNA levels resulted in a significant reduction in GSTM1 immunoreactive protein, Western analysis was performed in male, 16-week-old SHRSP\textsubscript{GLA}, SP.WKY\textsubscript{GLA}2c\textsuperscript{*}, and WKY\textsubscript{GLA} strains (Figure 3A). A single immunoreactive protein band of \(\approx 26\) kDa was detectable in each of the strains using GSMT1 antibody. Densitometry of these immunoblots was performed and confirmed significant reductions of GSTM1 protein levels in the SHRSP\textsubscript{GLA} (\(n=3\); SHRSP\textsubscript{GLA} 2.3\(\pm\)0.5 versus SP.WKY\textsubscript{GLA}2c\textsuperscript{*} 4.4\(\pm\)0.6; 95\% confidence interval [CI], 0.7, 3.5; SHRSP\textsubscript{GLA} 2.3\(\pm\)0.5 versus WKY\textsubscript{GLA} 4.8\(\pm\)0.1; 95\% CI, -1.0, -4.1; \(F=18.2; P=0.005\)). Nitrotyrosine immunoreactivity was used as a measure of oxidative stress in the kidneys of SHRSP\textsubscript{GLA} (Figure 3B). Western analysis clearly identified immunoreactive bands corresponding to nitrotyrosine at several sizes extracted from rat kidney. These bands were consistently more intense in kidneys from SHRSP\textsubscript{GLA} than SP.WKY\textsubscript{GLA}2c\textsuperscript{*} and WKY\textsubscript{GLA} when normalized to Gapdh (Figure 3B).
GSTM1 Is Implicated in Oxidative Stress in SHRSP

We investigated renal expression and cellular localization of the protein encoded by Gstm1 together with parallel measurements of oxidative stress in renal and vascular tissues. We identified previously Gstm1 as a positional candidate gene for hypertension in the SHRSPGla using a combination of congenic strain construction and microarray expression profiling. Using qRT-PCR, we observe a significant reduction in renal Gstm1 mRNA levels in 5-week-old SHRSPGla compared with SP.WKYGla2c* congenic and WKYGla parental rats at a time point before the onset of significant hypertension in the SHRSPGla. This suggests that altered gene expression of Gstm1 may contribute directly to the pathogenesis of hypertension and is not an adaptive response caused by long-term differences in blood pressure.

Discussion

Recent developments from the rat genome consortium have allowed the first assessment of the frequency of SNPs within rat strains, and many have focused on SNPs in protein coding regions because these polymorphisms are most likely to contribute to phenotypic differences. The identification of Arg 202 in the SHRSPGla and His 202 in the WKYGla is intriguing; however, it is clear that amino acid 202 is principally conserved as arginine in many of the GSTM enzymes. Furthermore, the modeling of the R202H amino acid change showed no perturbations other than the breaking of a local salt-bridge, and therefore, the substitution would be unlikely to affect enzyme activity. However, it is clear that SNPs in noncoding regions may also have functional effects, as suggested by evolutionary conservation and the mapping of human disease susceptibility to noncoding regions. The frequency and organization of SNPs identified in the Gstm1 regulatory sequences are consistent with the observation that SNPs are not randomly distributed across loci, reflecting regional variation with only a small proportion potentially disrupting transcription factor binding sites. Using in silico strategy, 3 of the 13 SNPs identified were mapped to conserved promoter regions across rat, mouse, and human Gstm genes, including 2 AML1 and HFN4 transcription factors and may account for differences of expression between SHRSPGla and WKYGla. The expression of several other glutathione S-transferases and cytochrome P450s has been shown to be regulated by HNF transcription factors. These results are further supported by our new findings that the Gstm1 gene sequence and its mRNA expression are very similar between the SHRSPGla and the SHR (Harlan).

The significant reduction in Gstm1 protein levels and immunohistochemical localization present an intriguing hypothesis. The distal part of the nephron, composed of the distal convoluted tubule and collecting duct, has the primary role in determining net NaCl and K+ balance. One of the principal functions of the collecting tubule is the reabsorption of NaCl. This activity is regulated at least partially by NO. A reduced bioavailability of NO has been postulated to

**Immunohistochemistry Analysis**

The relative levels and localization of GSTM1 were further analyzed using immunohistochemistry in kidney sections obtained from 16-week-old male SHRSPGla and WKYGla rats. Figure 4A through 4D shows that expression is limited to tubular epithelium and highlights the difference in intensity levels between kidneys from SHRSPGla and WKYGla. The specific site within the nephron was determined by performing colocalization studies with AQP2, a selective marker of principal cells of the collecting ducts (Figure 4E). As can be seen in Figure 4F, there is a precise alignment of the staining patterns showing that the site of expression of GSTM1 is in the collecting tubule. Nonimmune IgG controls for GSTM1 and AQP2 are shown in Figure 4G and 4H, respectively.

**Superoxide Levels in Kidney and Vascular Tissues**

Superoxide levels in the kidney medulla and cortex were significantly higher in SHRSP compared with WKY, with that of SP.WKYGla2c* being intermediate (n=3; SHRSP medulla 1813±114; cortex 1878±205; WKY medulla 335±240; cortex 524±368; SP.WKYGla2c* medulla 1276±337; cortex 957±176 cpm/mg protein; ANOVA medulla F=9.48; P=0.01; ANOVA cortex F=7.14; P=0.03). As can be seen in Figure 4F, there is a precise alignment of the staining patterns showing that the site of expression of GSTM1 is in the collecting tubule. Nonimmune IgG controls for GSTM1 and AQP2 are shown in Figure 4G and 4H, respectively.
contribute to the development of hypertension, but the underlying mechanism has not been resolved, particularly in light of the fact that a number of groups have shown either no change or enhanced expression of NO synthase in the kidneys of hypertensive rats. Welch et al demonstrated that the SHR kidney has increased immunoreactive expression of nitrotyrosine. Because antioxidants such as membrane-permeant superoxide dismutase blunt exaggerated tubular glomerular filtration responses, it has been proposed that the SHR kidney has an increased production of superoxide, which decreases the bioavailability of locally formed NO. We now suggest that the SHR kidney has an increased production of superoxide, which decreases the bioavailability of locally formed NO. We now suggest that it is a reduction in antioxidant defenses, evident by a reduced expression of \textit{Gstm1}, which decreases the bioavailability of superoxide, which decreases the bioavailability of NO in the renal medulla and cortex. Furthermore, parallel increase in superoxide levels in the SHRSP vasculature is consistent with the reduction in vascular NO bioavailability observed in clinical and experimental models of hypertension. It is tempting to hypothesize that altered expression of \textit{Gstm1} or genes belonging to antioxidant defense mechanisms may contribute to this increase in vascular superoxide in a manner analogous to the proposed mechanism in the kidney. Indeed, human subjects who have a GSTM1 null genotype may be more prone to atherosclerosis and endothelial dysfunction, particularly in the presence of compounding risk factors such as smoking.

**Perspectives**

We propose that \textit{Gstm1} may represent a novel candidate gene for hypertension and oxidative stress. The oxidative stress pathway has been implicated not only in the pathogenesis of endothelial dysfunction and hypertension but also all other modifiable cardiovascular risk factors, including diabetes, obesity, and hyperlipidemia. Therefore, imbalance between generation of ROS and endogenous antioxidant defenses might be central to several cardiovascular phenotypes. The
existence of 4 human orthologues will make it possible to translate these data directly to human studies.

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