Glutathione S-Transferase-µ1 Regulates Vascular Smooth Muscle Cell Proliferation, Migration, and Oxidative Stress

Yanqiang Yang, Kelly K. Parsons, Liqun Chi, Sandra M. Malakauskas, Thu H. Le

Abstract—Glutathione S-transferase-µ1, GSTM1, belongs to a superfamily of glutathione S-transferases that metabolizes a broad range of reactive oxygen species and xenobiotics. Across species, genetic variants that result in decreased expression of the Gstm1 gene are associated with increased susceptibility for vascular diseases, including atherosclerosis in humans. We previously identified Gstm1 as a positional candidate in our gene mapping study for susceptibility to renal vascular injury characterized by medial hypertrophy and hyperplasia of the renal vessels. To determine the role of Gstm1 in vascular smooth muscle cells (VSMCs), we isolated VSMCs from mouse aortas. We demonstrate that VSMCs from the susceptible C57BL/6 mice have reduced expression of Gstm1 mRNA and its protein product compared with that of the resistant 129 mice. After serum stimulation, C57BL/6 VSMCs proliferate and migrate at a much faster rate than 129 VSMCs. Furthermore, C57BL/6 VSMCs have higher levels of reactive oxygen species and exhibit exaggerated p38 mitogen-activated protein kinase phosphorylation after exposure to H2O2. To establish causality, we show that knockdown of Gstm1 by small interfering RNA results in increased proliferation of VSMCs in a dose-dependent manner, as well as in increased reactive oxygen species levels and VSMC migration. Moreover, Gstm1 small interfering RNA causes increased p38 mitogen-activated protein kinase phosphorylation and attenuates the antiproliferative effect of Tempol. Our data suggest that Gstm1 is a novel regulator of VSMC proliferation and migration through its role in handling reactive oxygen species. Genetic variants that cause a decremental change in expression of Gstm1 may permit an environment of exaggerated oxidative stress, leading to susceptibility to vascular remodeling and atherosclerosis. (Hypertension. 2009;54:1360-1368.)

Key Words: glutathione S-transferase-µ1 • vascular smooth muscle cells • proliferation • migration • reactive oxygen species

Glutathione S-transferase (GST)-µ1, GSTM1, belongs to a superfamily of glutathione S-transferases that metabolizes a broad range of reactive oxygen species (ROS) and xenobiotics. There are 8 distinct classes of soluble GSTs that have been identified thus far according the substrate specificity, chemical affinity, structure, and kinetic behavior of the enzyme.1,2 GSTM1 belongs to the Mu (µ) class, and is 1 of the 5 µ class of GST genes in humans.3 In mice there are 7 Gstm genes,4,5 and Gstm1 is the most abundantly expressed among all of the Gstm genes in the kidney.6

In humans, a GSTM1 deficiency state exists in those carrying the null allele, GSTM1(0), that arose from a recombination event during evolution between 2 highly homologous regions flanking this locus, resulting in deletion of a 20-kb segment.7,8 The prevalence of subjects carrying this allele in the homozygous state ranges from 30% to 50% in different human populations.9 The significance of this genetic variation in human was first recognized in cancer studies demonstrating that patients carrying the GSTM1(0) allele were at increased risks for colon and lung cancers.10,11 In subsequent studies undertaken in cardiovascular disease, subjects homozygous for the GSTM1(0) allele were shown to have increased risks of hypertension12 and atherosclerosis,13 as well as increased DNA alterations in atherosclerotic lesions of the abdominal aorta.14 Despite the circumstantial evidence for a role of the variant of the GSTM1 gene in human disease, the exact contribution of this gene in the vasculature has not been well characterized.

In genetic studies of hypertension, genome-wide scans performed on several rat crosses identified QTLs involved in blood pressure regulation.15 Subsequently, congenic strains were derived to isolate 1 of these QTLs, and comparisons of microarray expression profiling of a congenic strain versus the original parental strains identified Gstm1 as a positional and functional candidate gene.16 Gstm1 mRNA expression was found to be reduced in the stroke-prone spontaneously hypertension rat (SHR) compared with the congenic and normotensive Wistar Kyoto (WKY) rats.17 The differences in mRNA expression levels were reflected at the protein level in the kidney and were inversely correlated with renal levels of ROS, suggesting that the pathophysiological role Gstm1 in hypertension is likely to involve defense against oxidative stress.17

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From the Department of Medicine (Y.Y., K.K.P., L.C.), Duke University Medical Center, Durham, N.C.; Department of Medicine (S.M.M.), University of Alabama at Birmingham, Birmingham, Ala; Department of Medicine (T.H.L.), University of Virginia, Charlottesville, Va.
Correspondence to Thu H. Le, University of Virginia, Box 800133, Charlottesville, VA 22908. E-mail thl4t@virginia.edu
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1360
In previous studies, we identified a single locus, Msrvl1, on chromosome 3 that was linked to renal vascular injury in a mouse model, the angiotensin II type 1A receptor–deficient model, with vascular lesions characterized by medial hypertrophy and hyperplasia of the renal vasculature. As part of our screening process for possible positional candidate modifier gene(s), we first prioritized candidate genes as those that are differentially expressed between the susceptible (C57BL/6) and resistant (129S6) mouse strains. Within the critical interval of the Msrvl1 region containing ~50 genes, only Gstm1 exhibited robust and statistically significant differences in expression levels between the 2 strains. We found that the resistant 129 mice had twice the level of Gstm1 expression compared with the susceptible C57BL/6.

Taken together, the evidence suggests that Gstm1 plays a role in vascular homeostasis. The naturally occurring strain variation in Gstm1 expression would provide a powerful model for testing the functional role of Gstm1 in the vasculature. In this report, we determined strain differences in Gstm1 expression in vascular smooth muscle cells (VSMCs) and assessed the role of Gstm1 in VSMC proliferation, ROS production, and migration.

Materials and Methods

An expanded Materials and Methods section can be found in the online Data Supplement at http://hyper.ahajournals.org.

Primary VSMC Culture

VSMCs from aortas of 3- to 4-week-old wild-type C57BL/6 (Jackson Laboratory, Bar Harbor, ME) and 129S6 (Taconic) mice were isolated by enzymatic digestion using collagenase, 1.5 mg/mL (Sigma), while suspended in DMEM (Gibco Laboratories) containing L-glutamine, HEPES, penicillin, and streptomycin. Cells were washed and grown in DMEM supplemented with 10% fetal bovine serum, penicillin (100 U/mL), and streptomycin (100 µg/mL) in 75-cm² Corning tissue culture flasks at 37°C in a humidified environment of 5% CO₂ and air.

Cell Proliferation Assays

Cell proliferation was measured using both 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) and 5-bromodeoxyuridine assays.

Real-Time RT-PCR

Total RNA was isolated from VSMCs or aorta by RNeasy mini kit (Invitrogen). One microgram of DNAse I–treated RNA sample was reverse transcribed using SuperScript II First-Strand Synthesis System for RT-PCR (Gibco BRL) in a total reaction volume of 20 µL. Real-time RT-PCR was performed as described in the online Supplemental Data.

DHE Staining

VSMCs were seeded at 2 × 10⁴ cells per well in 24-well plates and allowed to grow overnight. Medium was then removed and cells were rinsed twice with Hank’s HEPES buffer solution, followed by the addition of 1 mL of HBSS to each well with 2.0 µmol/L of dihydroethidium (DHE). Cells were incubated at 37°C for 30 minutes in the dark. DHE was removed, and cells were rinsed with HBSS twice, followed by the addition of fresh HBSS. Fluorescence microscopy was performed after 30 minutes of incubation.

Detection of H₂O₂ With 2’/7’Dichlorofluorescein Diacetate

H₂O₂ in VSMCs was measured using 2’/7’-dichlorofluorescein diacetate (DCF-DA; Sigma) probe. VSMCs at 5 × 10⁴ per well were plated in a 96-well plate and allowed to grow overnight in DMEM. The medium was then removed, and the cells were rinsed twice with Hank’s HEPES buffer solution. Cells were then incubated with 10 µM of DCF-DA at 37°C with 5% CO₂ for 30 minutes. The fluorescent signal was detected by a microplate reader (FLUOstar, OPTIMA, BMG Labtech) at 485-nm excitation and 535-nm emission.

Detection of Superoxide by Lucigenin Assay

Production of superoxide was measured by lucigenin-enhanced chemiluminescence response. Briefly, for cultured VSMCs, cell suspension was created by detachment with 0.02% trypsin and 0.02% EDTA. Cells were washed with modified Krebs buffer containing NaCl (130 mmol/L), KCl (5 mmol/L), MgCl₂ (1 mmol/L), CaCl₂ (1.5 mmol/L), K₂HPO₄ (1 mmol/L), and HEPES (20 mmol/L; pH 7.4) and were resuspended in Krebs buffer with 1 mg/mL of BSA containing lucigenin (0.25 mmol/L). The cell concentration was then adjusted to 1 × 10⁴/mL. To measure ROS production, the cell suspension was transferred into polypropylene tubes and assessed in a luminometer (OPTOCOMP I, GEM Biomedical, Inc). Counts were obtained at 10 minutes of incubation. Background counts determined in cell-free preparations were subtracted from the total count.

RNA Interference and Cell Transfection

High-performance purity grade (≥90% pure) small interfering RNAs (siRNAs) against Gstm1 (Gstm1-siRNA) were obtained from Ambion, Inc. siRNAs, with a nonsilencing oligonucleotide sequence (nonsilencing siRNA) that does not recognize any known homology to mammalian genes, are used as a negative control (control-siRNA). VSMCs were seeded at a density of 5 × 10⁴ cells well in 6-well plates and grown in DMEM containing 10% FCS. One day after seeding, cells are transfected with 100 pmol of control-siRNA or 100 pmol of Gstm1-siRNA using lipofectomine NucFect Transfection Reagent (Qiagen Inc) according to the manufacturer’s instructions. Seventy-two hours posttransfection, the cells were then analyzed by Western blot, cell proliferation, DHE staining and lucigenin, or migration assays.

Western Blot

The method used has been described previously. VSMCs were lysed in radioimmunoprecipitation assay buffer with protease inhibitors. Rabbit anti-GSTM1 (generous gift of Dr John Hayes) was used at 1:2000 dilution.

Cell Migration Assay

Cell migration was assessed using 24-well plates with Transwell inserts (8.0 µm pore; Costar), as described previously.

Statistical Analysis

Data are expressed as mean±SE. For comparisons between strains, n = 3 aortas for each strain of 129 and C57BL/6 used for isolation of primary VSMCs. All of the experiments were performed in triplicate and repeated 3 to 4 times. Student t test was used for all of the comparisons between 2 groups, and ANOVA was used for comparisons among 3 groups.
Results

Strain Differences in Expression of \textit{Gstm1} in VSMCs

On the basis of our previous gene mapping study demonstrating that \textit{Gstm1} is a candidate gene for susceptibility to renal vascular pathology,\textsuperscript{18} we asked whether expression of \textit{Gstm1} in VSMCs is different between the susceptible C57BL/6 strain versus the resistant 129S6 (129) strain. We isolated VSMCs from the aorta from wild-type C57BL/6 and 129 mice and grew them in culture. By real-time RT-PCR and Western analysis, \textit{Gstm1} mRNA and protein levels, respectively, were significantly decreased by 50\% in C57BL/6 VSMCs compared with 129 VSMCs (Figure 1).

Strain Differences in VSMC Proliferation and ROS Production

While culturing primary VSMCs from the 2 different strains (n=3 each), we noted that C57BL/6 VSMCs in culture proliferated and reached confluence at a much faster rate than 129 VSMCs. To better quantitate their growth rate, we measured VSMC proliferation. By MTS assay (Figure 2A), starting at the same cell number plating on day 0, with 10\% serum, at 48 and 72 hours, C57BL/6 VSMCs proliferated 2 times faster than 129 VSMCs (P\leq0.03). To confirm our observations, we also determined cell proliferation using the 5-bromodeoxyuridine cell proliferation assay (Figure 2B). This assay also demonstrated a higher cell proliferation rate in the C57BL/6 VSMCs, and the difference was highly statistically significant by 24 hours.

Because a previous study showed that lower renal expression of \textit{Gstm1} in stroke-prone SHRs was associated with higher renal ROS levels compared with WKY rats,\textsuperscript{17} we examined ROS levels in our 2 different murine VSMC lines. As an indirect measure of superoxide levels, we performed DHE staining of VSMCs in culture. At equal cell density by 4,6-diamidino-2-phenylindole staining, DHE staining was significantly increased in C57BL/6 VSMCs compared with 129 VSMCs, suggesting increased superoxide levels (Figure 3A). We also assessed ROS (H$_2$O$_2$) generation in VSMCs using a DCF-DA probe. By detection of dichlorofluorescein fluorescence (Figure 3B) over a 50-minute interval, C57BL/6 VSMCs have much higher levels of ROS production compared with 129 VSMCs.

Reduction in \textit{Gstm1} Causes Increased Cell Proliferation and ROS Production

To determine whether the difference in expression of \textit{Gstm1} between the 2 different strains of VSMCs could directly cause differences in cell proliferation and ROS production, we used siRNA targeting \textit{Gstm1} (Ambion, Inc). Using \textit{Gstm1}-siRNA, we successfully knocked down \textit{Gstm1} mRNA
levels in both 129 and C57BL/6 VSMCs by 60% to 80% \((P<0.05; \text{Figure 4A})\). We next examined the effect of Gstm1 knockdown by siRNA on cell proliferation. As shown in Figure 4B (top), in both 129 and C57BL/6 VSMCs, compared with scrambled siRNA, Gstm1-siRNA resulted in significantly increased VSMC proliferation. Specifically, after 72 hours, 129 VSMCs treated with Gstm1-siRNA had similar proliferation rates as C57BL/6 cells treated with scrambled-siRNA. Moreover, C57BL/6 cells treated with Gstm1-siRNA had significantly higher proliferation rates than cells treated with control-siRNA. Western blotting (Figure 4B, bottom) demonstrated that GSTM1 protein levels in 129 Gstm1-siRNA treated cells were very similar to that of the C57BL/6 control-siRNA condition. Furthermore, C57BL/6 cells treated with Gstm1-siRNA had barely detectable GSTM1 by Western analysis. Our data suggest that Gstm1 exerts an antiproliferative effect in a dose-dependent manner.

We next determined whether knockdown of Gstm1 expression in 129 VSMCs would affect ROS production. By DHE

**Figure 3.** Strain differences in ROS production. A, DHE staining. At relatively equal cell density determined by 4',6-diamidino-2-phenylindole nuclear (DAPI) staining (bottom left and right panels), there is very low level of DHE staining in the 129 cells (top left). However, in C57BL/6 VSMCs, there is dramatically higher level of DHE staining (top right), suggesting higher levels of superoxide. B, DCF-DA assay. The dichlorofluorescein fluorescent signal, a measure of \(\text{H}_2\text{O}_2\) levels, is significantly higher in C57BL/6 VSMCs vs 129; \(P=0.001; n=3\) each; performed in triplicate in 3 experiments.

**Figure 4.** Effect of knockdown of Gstm1 by siRNA. A, Gstm1-siRNA successfully decreased Gstm1 expression in both 129 and C57BL/6 VSMCs by 60% to 80%; \(P<0.0005\) vs control (C-siRNA); \(n=3\) each; performed in triplicate. B, Top, Gstm1-siRNA resulted in significantly increased VSMC proliferation. Seventy-two hours after siRNA transfection, 129 VSMCs treated with Gstm1-siRNA had significantly higher proliferation rate as 129 cells treated with control-siRNA \((P=0.008, 129 \text{ control-siRNA} \text{ vs } 129 \text{ Gstm1-siRNA})\) but similar proliferation rate as C57BL/6 cells treated with control-siRNA \((P=0.40, 129 \text{ Gstm1-siRNA} \text{ vs } \text{C57BL/6 \text{ Control-siRNA}})\). C57BL/6 cells treated with Gstm1-siRNA had an even higher proliferation rate than cells treated with control-siRNA \((P=0.02, \text{C57BL/6 control-siRNA} \text{ vs C57BL/6 Gstm1-siRNA})\). \(n=3\) for each condition, performed in triplicate, in 3 separate experiments. Bottom, Western analysis demonstrates successful knockdown of the enzyme in both cell lines vs control-siRNA. Of note, Gstm1-siRNA reduced GSTM1 protein expression of 129 VSMCs to similar level as seen in C57BL/6 cells treated with control-siRNA. C57BL/6 cells treated with Gstm1-siRNA had barely detectable GSTM1 protein levels.
staining (Figure 5A), Gstm1-siRNA causes a marked increase in superoxide production compared with control-siRNA. To better quantitate the differences, we performed an assay using lucigenin chemiluminescence. As shown in Figure 5B, in 129 VSMCs, Gstm1-siRNA caused a significant increase in superoxide production as measured by increased luminescence levels in VSMCs compared with control-siRNA.

**Strain Differences in p38 Phosphorylation and Effect of Gstm1 Knockdown After Exposure to Hydrogen Peroxide**

ROS are thought to serve as second messengers that activate downstream kinases, such as p38 mitogen-activated protein kinase.21 With 2 distinct VSMC lines with naturally occurring strain-dependent expression levels of Gstm1, we posited that the 129 VSMCs with higher expression levels of Gstm1 would be more resistant than C57BL/6 VSMCs to p38 phosphorylation after H2O2 treatment. As expected, VSMCs from 129 demonstrated significantly less p38 phosphorylation compared with C57BL/6 after H2O2 exposure (Figure 6A). We next determined the effect of Gstm1 knockdown on p38 phosphorylation in 129 VSMCs. As shown in Figure 6B, Gstm1 siRNA resulted in a significant increase in p38 phosphorylation in 129 VSMCs after treatment with H2O2 compared with untransfected wild-type cells or cells transfected with control-siRNA.

Reduction of Gstm1 Attenuates Antiproliferative Effects of Tempol

To determine whether the effect of Gstm1 on cell proliferation is mediated through its regulation of superoxide levels, we next assessed the effect of Tempol with and without Gstm1 knockdown. As shown in Figure 7A, Gstm1-siRNA blunted the inhibition of cell proliferation by Tempol by 50% at both a lower dose (2.5 mmol/L) and a higher dose (5 mmol/L) of Tempol. Furthermore, with Gstm1-siRNA, the higher dose of Tempol was required to achieve a similar degree of inhibition of cell proliferation as in the lower dose with control-siRNA. Conversely, Tempol attenuated the proliferative effect of Gstm1-siRNA (Figure 7B). Our data suggest that the effect of Gstm1 is mediated, at least in part, through its regulation of superoxide levels; loss of GSTM1 results in decreased clearance of superoxide, increased oxidative stress, and, hence, increased VSMC proliferation.

**Effects of Gstm1 on VSMC Migration**

It has been shown that ROS are key mediators for platelet derived-growth factor (PDGF) signal transduction, because
blockade of H$_2$O$_2$ accumulation by catalase inhibits PDGF-induced migration of VSMCs. Because reduction of Gstm1 results in increased superoxide and H$_2$O$_2$ levels, we queried whether Gstm1, through its role in handling oxidative stress, regulates VSMC migration. We first examined migration rates between C57BL/6 and 129 VSMCs with low and high Gstm1 expression, respectively. As shown in Figure 8A, the cell migration rates in untreated cells were similar between 129 and C57BL/6 cells. However, after stimulation with PDGF, cell migration rate was much faster in C57BL/6 VSMCs than in 129 VSMCs. To determine whether this difference might be contributed by differences in Gstm1 expression, we used Gstm1-siRNA in 129 VSMCs to demonstrate causality. After Gstm1 knockdown with siRNA, 129 VSMCs migrated at a much faster rate compared with control-siRNA and untransfected conditions (Figure 8B).

**Discussion**

Our studies demonstrate that genetic variation in expression of Gstm1 is associated with differences in VSMC proliferation, ROS production, and cell migration. Furthermore, we establish a functional role of Gstm1, as we find that reduction of Gstm1 in VSMCs using siRNA directly causes increased cell proliferation, oxidative stress, and migration. Our findings of differences in proliferation between the 129 and C57BL/6 murine VSMCs are very similar to the earlier studies reporting that VSMCs isolated from thoracic aorta of the stroke-prone SHR strain compared with the congenic and normotensive WKY rats. More recently, Gstm1 was identified as a positional and functional candidate gene for hypertension and was found to be reduced in kidney tissues from the SHR strain proliferate faster than those from the normotensive WKY rats. We suggest that the differences in cell proliferation between these 2 rat strains are also attributed, at least in part, to differences in Gstm1 expression.

One important consideration is whether the effect that we observe is specific to Gstm1 or is attributed to combined effects of other differentially expressed Gstm genes. In this regard, we find that, in the mouse aorta, along with Gstm1, Gstm4 and Gstm5 are also more significantly highly expressed in 129 than in C57BL/6 mice. However, similar to previous studies describing the relative expression of all of the Gst genes in the kidney, we also find that, in the aorta in both strains, the expression of the Gstm1 gene, relative to GAPDH, is significantly and several times higher than any
other Gstm genes (Figure S1), suggesting that, in the vasculature, Gstm1 plays the most dominant role within its class, and perhaps among all of the Gst classes, because GSTM1 is the most predominant glutathione S-transferase in both the mouse kidney and lung, accounting for 45% and 60% of the total GST content, respectively. Moreover, we found no change in the expression of the other 5 Gstm genes (Gstm2 to Gstm6) in VSMCs after reduction of Gstm1 by siRNA knockdown (data not shown). This suggests that the effect that we observe in vitro is specifically attributable to knockdown of Gstm1 and that there is no loss or compensatory changes from other Gstm genes that could account for our observations. It is worth noting here that, whereas Gstm7 has been reported, we have not been able to confirm expression of this gene using previously reported primers. We have redesigned primers for Gstm7 but have not successfully identified its expression in either mouse aortic tissues or isolated VSMCs.

Compared with the 129 strain, the C57BL/6 strain is more susceptible to atherosclerosis and ocular neovascularization. In our genetic model of angiotensin II type 1A receptor deficiency, we found that the C57BL/6 strain is more susceptible to renal vascular injury. Taken together, we speculate that the lower expression of Gstm1 in the vasculature in the C57BL/6 strain contributes to the generalized increased susceptibility to vascular injury and remodeling.

Approximately 30% to 50% of individuals in most human populations completely lack the activity of the detoxifying enzyme GSTM1. This results from homozygous inheritance of the GSTM1 null allele, GSTM1(0), that has a 20-kb segment deletion of the gene. The 3 genotypes, homozygously active GSTM1/GSTM1, heterozygously deficient GSTM1/GSTM1(0), and homozygously deficient GSTM1(0)/GSTM1(0), are associated with a trimodal distribution of glutathione-conjugator activity, with high, intermediate, and nonconjugators, respectively. Examination of public databases reveals that GSTM1 lies within a cluster with 4 other GSTM genes on human chromosome 1, and this region is a hot spot for gene copy number variation. Thus, it is likely that other GSTM1 polymorphisms may also contribute to expression differences of GSTM1 in humans. The exact polymorphism causing Gstm1 expression differences in stroke-prone SHR strains is unknown. We have sequenced the putative promoter region of the murine Gstm1 gene, as well as all of the exons, introns, and 3′ untranslated regions and have not detected any sequence variation between the 129 and C57BL/6 mouse strains. On the basis of our gene mapping study, it is likely that the regulating variant affecting Gstm1 expression, and that of Gstm4 and Gstm5, is cis-acting and lies in a yet-to-be-discovered regulatory region of the gene cluster.

In disease states such as atherosclerosis and arterial injury–induced neointimal hyperplasia, it is generally thought that a key component involves medial smooth muscle cell proliferation and migration into the arterial intima. We demonstrate here that VSMCs with lower Gstm1 expression, from either naturally occurring genetic variation or siRNA knockdown, have increased cell proliferation and migration rates. Our studies provide a possible mechanistic link for increased susceptibility to atherosclerosis in those carrying the GSTM1(0) allele.

The exact mechanism(s) by which Gstm1 influences cell proliferation, ROS production, and cell migration is unknown. We find that reduction of Gstm1 expression results in increased production of ROS, which are thought to function as second messengers that activate the phosphorylation of downstream kinases, such as p38 mitogen-activated protein kinase. We show that, with serum stimulation, Gstm1 directly and dose dependently affects VSMC growth; the lower the Gstm1 expression level, the higher the cell proliferation rate. Suh et al showed previously that serum–induced proliferation of VSMCs is mediated by Nox1, a member of the NADPH oxidase. NADPH oxidases are generally recognized to be the major contributor to ROS production in the vasculature. The prodigious effect on VSMCs by the hormones angiotensin II, PDGF, and thrombin, and the cytokine tumor necrosis factor α (TNF α) is thought to be via the common activation of NADPH oxidase. The result is an increased level of NADPH oxidase–derived superoxide that increases the activity of downstream kinases, such as p38 mitogen-activated protein kinase and Akt. We find that C57BL/6 VSMCs are more susceptible to p38 phosphorylation compared with 129 when exposed to H2O2. Furthermore, p38 phosphorylation is virtually undetectable in 129 VSCMs at baseline, or even after exposure to H2O2, but is significantly enhanced after exposure to H2O2 in the presence of Gstm1 siRNA. Taken together, we postulate that Gstm1 modulates the NADPH oxidase–induced signaling pathway, perhaps through its role in regulating ROS levels.

Whether GSTM1 has superoxide dismutase and/or catalase-like activity remains to be determined. In our studies using low doses of Tempol, the addition of Gstm1 siRNA resulted in reduced effectiveness of Tempol to suppress cell proliferation. Moreover, Tempol attenuates the proliferative effect of Gstm1 siRNA. One alternative explanation is that GSTM1 reduces the effectiveness of Tempol simply through its metabolism of the compound. However, in 129 VSMCs, Gstm1-siRNA caused significant p38 phosphorylation after exposure to H2O2. These observations suggest that GSTM1 directly regulates ROS levels. It is possible that GSTM1 regulates intracellular ROS through its well-known catalytic activity in intracellular glutathione conjugation and metabolism, whereby regulating the redox state. In addition to its active catalytic site, GSTM1 also has a functional noncatalytic domain that inhibits activation of the apoptosis–signaling–regulating kinase 1 (ASK1)–p38 signaling pathway. Under normal conditions, GSTM1, via its noncatalytic site, binds to ASK1, thereby inhibiting ASK1 activation. In conditions of stress, such as heat shock or exposure to H2O2, GSTM1 is thought to be released from ASK1, leading to activation of ASK1 and downstream kinases. Our finding that Gstm1 expression regulates p38 phosphorylation is consistent with these earlier observations. Another possibility is that Gstm1 modulates VSMC proliferation and migration through its role in protection against accumulation of lipid peroxidation products that are now recognized to influence cell proliferation and migration. It has been demonstrated that human subjects homozygous for GSTM1(0) have
higher plasma levels of malondialdehyde, a product of lipid peroxidation.58

Perspectives
Gene mapping and association studies have demonstrated that, across human, rat, and murine species, genetic variations of the Gstm1 gene are associated with cardiovascular diseases. The present study describes a novel function of Gstm1 in the regulation of VSMC proliferation and migration, perhaps through its role in handling oxidative stress. These findings implicate that genetic variants that cause even a modest decremental effect in the expression of the Gstm1 gene provide a permissive environment of exaggerated oxidative stress, leading to enhanced susceptibility to vascular remodeling and atherosclerosis. Gstm1 may serve as a modifier of phenotype in disease states.

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Disclosures
None.

References


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Glutathione -S-Transferase μ 1 Regulates Vascular Smooth Muscle Cell Proliferation, Migration, and Oxidative Stress

Yanqiang Yang, MD, PhD¹, Kelly K. Parsons, PhD¹, Liqun Chi, BA¹, Sandra Malakauskas, PhD, MD², and Thu H. Le, MD³

1. Department of Medicine, Duke University Medical Center, Durham, NC, 27710
2. Department of Medicine, University of Alabama at Birmingham, Birmingham, AL, 35294
3. Department of Medicine, University of Virginia, Charlottesville, VA 22908

Running title: Gstm1 modulates intracellular oxidative stress

Author for Correspondence:

Thu H. Le, MD
University of Virginia
Box 800133
Charlottesville, VA 22908
Phone: 434-982-1063
E-mail: thu.le@virginia.edu
Expanded Materials and Methods

Primary vascular smooth muscle cell culture. Cells were checked for a characteristic hill and valley pattern, and identity was verified by immunostaining using monoclonal anti-smooth muscle actin antibody. The incubation media was changed twice weekly. Passage was made when cells were near confluent. For passaging, cells were detached with 0.05% trypsin-0.53 mM EDTA (GIBCO Laboratories). Cells from same and low passages (3-7) were used when making comparisons between strains and control and siRNA conditions. All studies were performed with serum unless otherwise noted.

Cell proliferation assays. Cell proliferation was measured using both MTS and BrdU assays. For MTS assay, VSMCs were seeded in 96 wells plate at 1x10^3 cells per well in triplicates and incubated at 37°C in humidified, 5% CO2 atmosphere for 24-72h. To measure cell viability, a freshly prepared PMS-MTS solution of 20 µL was added to the each well and incubated for 1-3h. The results were recorded by an ELISA plate reader at a wave length of 490nm. For BrdU proliferation assay, the determination of the incorporation of 5-bromo-2'-deoxyuridine (BrdU) into VSMCs was performed using the BrdU proliferation kit (Calbiochem) according to the manufacturer's instructions. Briefly, VSMCs were seeded in 96-well plates at 1x10^3/well, then incubated with BrdU (10 µM) for 2 hours, 24h, 48h or 72h after transfection. Cells were fixed and BrdU was labeled with a peroxidase-conjugated anti-BrdU antibody followed by addition of a peroxidase substrate. The reaction was stopped by adding 100 µL of stop solution and optical density at 450 nm and 540 nm was measured in each well with a microplate reader (FLUROstar Optima, BMG Labtech, Germany).

Western Blot. VSMC were lyzed in RIPA buffer with protease inhibitors. Protein concentrations were determined by the BCA assay (Bioassay Systems). Twenty (20) µg of total protein of each sample is loaded onto 10% SDS-PAGE, and transferred to PVDF membrane (Invitrogen). Membranes were blocked with 5% nonfat milk in Tris Buffered Saline including 0.1% Tween-20 (TBST buffer), and incubated overnight at 4°C with rabbit anti-GSTM1 (generous gift of Dr. John Hayes (1)) at 1:2000 dilution, followed by incubation for 1 h at room temperature with the horseradish peroxidase-conjugated secondary antibodies. The signals were detected by enhanced chemiluminescence (GE Healthcare).

To detect the effect of Gstm1 on p38 activation, VSMCs were treated with H2O2 2mM for 30 min at 37°C and 5 % CO2. The cells were then lysed in RIPA buffer with protease and phosphatase inhibitor cocktail (Sigma). Phospho-p38 MAP kinase antibody (Cell Signaling) at 1:1000 dilution was used to detect p38 activation, and the membrane was re-probed with p38 antibody at 1:1000 dilution (Cell Signaling) and anti-Tubulin antibody at 1:5000 dilution (Santa Cruz Biotechnology).
**Real-time RT-PCR.** Real-time RT-PCR was performed using primers listed in Table S1. Fluorescence detection was accomplished using Sybr Green and the iCYcler system (Bio-Rad). *Gstm* mRNA expression levels were normalized against quantified GAPDH mRNA expression. Primer sequences for *Gstm1*, and *Gstm3-7* listed in Table S1 were obtained from work published by Chanas et al. (2). Due to non-specificity in our hands, primers for *Gstm2* were re-designed by our laboratory and are listed in Table S1.

**Table S1. Real-time RT-PCR. Oligonucleotide primers for *Gstm* 1 – 7 genes**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer Sequence</th>
<th>Product Size (BP)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gstm1</td>
<td>Forward: 5'-CCTATGATACCTGGATACCTGGAAACG-3’ &lt;br&gt;Reverse: 5'-GGAGCGTCACCATGGTG-3'</td>
<td>112</td>
</tr>
<tr>
<td>Gstm2</td>
<td>Forward: 5'-GCACAACCTGTGTGGAGAGA-3’ &lt;br&gt;Reverse: 5'-GTAGCAAACCATGGGCAACT-3'</td>
<td>100</td>
</tr>
<tr>
<td>Gstm3</td>
<td>Forward: 5'-TATGACACTGGGCTATTGGAACAC-3’ &lt;br&gt;Reverse: 5'-GGGCATCCCCCATGACA-3'</td>
<td>109</td>
</tr>
<tr>
<td>Gstm4</td>
<td>Forward: 5'-AGGCTATGGATGCTCACAATCAG-3’ &lt;br&gt;Reverse: 5'-TCCAGGGAGCTGCTCACA-3'</td>
<td>74</td>
</tr>
<tr>
<td>Gstm5</td>
<td>Forward: 5'-GCTGGACGTGAAATCTCTCAGCTA-3’ &lt;br&gt;Reverse: 5'-CGTTACTCGGTGATCTTCTTC-3'</td>
<td>85</td>
</tr>
<tr>
<td>Gstm6</td>
<td>Forward: 5'-CCTCCAGATCAGCTGAAACTCTA-3’ &lt;br&gt;Reverse: 5'-TCAAGGGACATCAGCAGGAAGCT-3'</td>
<td>94</td>
</tr>
<tr>
<td>Gstm7</td>
<td>Forward: 5'-TCCGTGTGGATATTCTGGAG-3’ &lt;br&gt;Reverse: 5'-CCTCATCATTCCAGGGAGTT-3'</td>
<td>125</td>
</tr>
</tbody>
</table>

**Cell migration assay.** Cell migration was assessed using 24-well plates with Transwell inserts (8.0 µm pore, Costar) as previously described (3). Quiescent VSMCs at concentration of 5 x 10⁴/well were added to the upper chamber, and PDGF-BB (25ng/mL) was added to lower chamber. The plate were incubated at 37°C, 5% CO₂ for 16 h, then filters were stained with 0.5% (w/v) crystal violet in 5% (v/v) ethanol. The unaccompanied VSMCs were removed by scraping. Crystal violet absorbed to migrated VSMCs was solubilized with 40% acetic acid, and VSMC number was estimated colorimetrically by a plate reader at 562 nm. Migration values for stimulated VSMCs were normalized to cognate unstimulated VSMCs to obtain “fold/basal” relative ratio.
References


Strain differences in expression of \textit{Gstm} genes 1 through 6 in aorta. Expression of each gene is expressed as relative to 129 \textit{Gstm}1 levels. Within strain, \textit{Gstm1} is most abundantly expressed among all \textit{Gstm} genes. Between strains, expression of \textit{Gstm1}, \textit{Gstm4}, and \textit{Gstm5} is lower in C57BL/6 compared to 129, \(*\, p < 0.05\); \(n = 3\) aortas from each strain, performed in triplicates. \textit{Gstm7} was not detectable in the aorta.