Glutathione S-transferase P protects against endothelial dysfunction induced by exposure to tobacco smoke

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Abstract

Exposure to tobacco smoke impairs endothelium-dependent arterial dilation. Reactive constituents of cigarette smoke are metabolized and detoxified by glutathione S-transferases (GSTs). Although polymorphisms in GST genes are associated with the risk of cancer in smokers, the role of these enzymes in regulating the cardiovascular effects of smoking has not been studied. The P isoform of GST, which catalyzes the conjugation of electrophilic molecules in cigarette smoke such as acrolein, was expressed in high abundance in mouse lung and aorta. Exposure to tobacco smoke for 3 days (5h/day) decreased total plasma protein. These changes were exaggerated in GSTP−/− mice. Aortic rings isolated from tobacco smoke-exposed GSTP−/− mice showed greater attenuation of acetylcholine-evoked relaxation than those from GSTP+/+ mice. Lung, plasma and aorta of mice exposed to tobacco smoke or acrolein (for 5h) accumulated more acrolein-adducted proteins than these tissues of mice exposed to air, indicating that exposure to tobacco smoke results in systemic delivery of acrolein. Relative to GSTP+/+ mice, modification of some proteins by acrolein was increased in the aorta of GSTP−/− mice. Aortic rings prepared from GSTP−/− mice inhaling acrolein (1 ppm, 5h/day for 3 days) or those exposed to acrolein in the organ bath showed diminished acetylcholine-induced arterial relaxation more strongly than GSTP+/+ mice. Acrolein-induced endothelial dysfunction was prevented by pretreating the aorta with N-acetylcysteine. These results indicate that GSTP protects against the endothelial dysfunction induced by tobacco smoke exposure and that this protection may be related
to the detoxification of acrolein or other related cigarette smoke constituents.

**Keywords:** acrolein, aldehydes, environmental cardiology, oxidative stress
Introduction

Extensive evidence demonstrates that cigarette smoking (25) or exposure to second-hand tobacco smoke (4) increases the risk for cardiovascular disease (CVD). Smoking causes an estimated 1.69 million deaths per year world-wide (17). Smoking is strongly and positively associated with an increase in the risk for myocardial infarction and sudden cardiac death and an increase in thrombosis and atherosclerosis (39). Smoking also induces endothelial dysfunction which is a characteristic feature of CVD. Nevertheless, pathophysiological mechanisms that mediate and exacerbate cardiovascular injury due to exposure to tobacco smoke remain poorly understood. In particular, the role of individual constituents of tobacco smoke in promoting CVD is not known and it is unclear whether metabolic pathways for the detoxification of chemicals in cigarette smoke modulate excessive CVD risk due to exposure to tobacco smoke.

Cigarette smoke contains 4,000 to 100,000 different chemicals and most of these are present in vanishingly small concentrations (39). In addition to nicotine, some of the most toxic cigarette smoke constituents present in high abundance include carbon monoxide, saturated and unsaturated aldehydes, vinylpyridine, hydrogen cyanide, particulate matter, and polyaromatic hydrocarbons (PAH). Of these, unsaturated aldehydes such as acrolein are likely to be particularly important because of their high reactivity and high cardiovascular toxicity (8). In most tissues, unsaturated aldehydes such as acrolein and crotonaldehyde are metabolized via conjugation by glutathione, a reaction that is catalyzed by glutathione S-transferases (GSTs). The GSTs are a large family of enzymes (GSTA, M, and P among others) that catalyze the conjugation of a
variety of electrophilic xenobiotics and have been implicated in the development of tumor resistance to anticancer drugs (23).

Results of several epidemiological studies suggest a link between polymorphism in GST genes and the risk of cancer due to smoking (38). The GSTM-null phenotype (16) and polymorphisms in the GSTP gene (11) have both been found to be associated with the elevated risk of bladder cancer in smokers. The association between GST genotype and lung cancer is less clear, because both positive and negative data have been reported (34; 38). Similarly, in some studies GSTM-null phenotype is associated with an increase in coronary artery disease in smokers (33), while in others the null phenotype was reported to be associated with a decrease in risk of myocardial infarction in smokers (46). Although GSTM participates in the metabolism of several PAH present in cigarette smoke, many of the small reactive carbonyls present in cigarette smoke, such as acrolein and crotonaldehyde, are preferentially conjugated by GSTP (8). Nonetheless, the role of GSTP in the cardiovascular effects of cigarette smoke has not been studied. Accordingly, the current study was designed to examine whether GSTP regulates smoking-induced endothelial dysfunction. In humans, passive smoking diminishes endothelium-mediated relaxation and long-term smoking is associated with impaired endothelium-dependent relaxation of conduit and coronary arteries (13; 14). Tobacco smoke exposure also decreases endothelium-dependent relaxation in rats and rabbits (29; 30) and increases arterial stiffness in mice (20). The results of our study show that deletion of the GSTP gene increases tobacco smoke-induced endothelial dysfunction and exacerbates acrolein-induced vascular injury.
suggesting that GSTP protects against smoking-induced endothelial dysfunction potentially by promoting the detoxification of acrolein and related electrophilic constituents of tobacco smoke.

**Materials and Methods**

*Mice:* Glutathione S-transferase-P1/P2 null mice were obtained from C. Henderson and R. Wolf (University of Dundee) in which both the GSTP genes were knocked out using a single construct (24). These mice grow and breed normally. Mice were treated according APS’s [Guiding Principles in the Care and Use of Animals](#) and all protocols were approved by University of Louisville IACUC.

*Exposure to Tobacco Smoke or Acrolein:* Male WT and GSTP-null mice (12-16 weeks old) were exposed to air (control) or to tobacco smoke for 1 or 3 days (5 h/day) and sacrificed either immediately after exposure (1 day) or at 16 h after the last treatment (3 day). Tobacco smoke was generated from Kentucky 2RF4 reference cigarettes (Tobacco Research Institute, University of Kentucky, Lexington, KY), which have a declared content of 2.45 mg nicotine each, with a 23 mm butt remaining after smoking. These cigarettes have a higher total content of acrolein and crotonaldehyde than the 1R4F cigarettes (15). The cigarettes were kept in standardized atmosphere humidified with 70 % glycerol and 30 % water for 48 h before use. Mice were exposed to tobacco smoke using a smoke chamber (model TE-10; Teague Enterprises, Woodland, CA). A mixture of sidestream (89 %) and mainstream (11 %) cigarette smoke (CS) was used in
tobacco smoke exposures. Each smoldering cigarette was puffed for 2 s, once every minute for a total of 8 puffs, at a flow rate of 1.05 l/min, to provide a standard puff of 35 cm³. Ten 2RF4 cigarettes were burned at one time for 5 h continuously per day. The total suspended particulate (TSP) level was 88.1±2.5 mg/m³ estimated from 5 separate 3-day exposures.

Acrolein atmospheres were generated from liquid acrolein (Sigma, >90 %) diluted in dH₂O (1:10) in a custom vapor system (Teague Inc.) using a primary chamber as a constant source. Acrolein vapors were diluted with HEPA-filtered room air in secondary chamber. Acrolein exposure concentration was continuously monitored using an in-line photoionization detector (ppbRAE+, Rae Industries, Sunnyvale, CA) prior to delivery via a cage insert vapor delivery unit (Teague Inc.) into a standard polycarbonate rat cage (16”x8.75“ x13.5”; ~31l) for exposures. Air or acrolein was distributed through a fine mesh screen at 3 lpm by delivery units with a cyclone-type top that distribute air within 10 % of the mean concentration at six locations in the cage. Exposure cages were placed partially over heating pads (~71 °F) to allow mice to select preferable temperature. In the first protocol, mice were exposed to 5 ppm acrolein for 5 h (4,944±44 ppb of 4 different exposures) and in the other to 1 ppm acrolein for 3 days (1,053±22 ppb of 9 different exposures).

Following exposure, the mice had free access to food (not during exposure) and water, after which they were sacrificed with sodium pentobarbital (0.1 ml; 40 mg/ml, i.p.). Blood was collected via cardiac puncture in Na₄·EDTA (0.2M; 16 µl/ml blood) containing tubes. Plasma protein and albumin levels were determined using Bradford
and bromocresol green reagents, respectively (Wako). Plasma ALT, AST (Infiniti) and CK, LDH (Promega) levels were measured using commercially available assay reagents in 96-well plates or with a Cobas Mira Plus 5600 Autoanalyzer (Roche). Organs (i.e., heart, kidney, liver, lung) were weighed, and then snap frozen in LN₂ for Western blotting or GST activity analyses and/or pieces of each were formalin-fixed (10% NBF) and processed for histology and immunohistochemistry. Total plasma HDL and LDL cholesterol, triglycerides, and phospholipids were determined using Cholesterol CII Enzymatic Kit (Wako) and L-Type TG-H Kit (Wako) using calibrated standards and a Cobas Mira Plus 5600 Autoanalyzer.

**Isolated Aorta and Vascular Reactivity:** Mice were anesthetized with sodium pentobarbital (0.15 ml, 40 mg/ml, i.p.) and given heparin (2,000 U, 0.2 ml, i.p.) and the aorta was removed via mid-ventral thoracotomy. Thoracic aorta segment ends were trimmed, one 4-mm (tobacco smoke and acrolein studies) or four 3-mm (naïve mice) rings were cut from each aorta, and rings were hung on stainless steel hooks in 15-ml water-jacketed organ baths in PSS bubbled with 95% O₂ and 5% CO₂ at 37 °C. The composition of the PSS was in mM: NaCl, 130; KCl, 4.7; MgSO₄·7H₂O, 1.17; KH₂PO₄, 1.18; NaHCO₃, 14.9; CaCl₂, 2.0; glucose, 5.0; pH 7.4. One hook was connected to an isometric strain gauge transducer (Kent Scientific; Litchfield, CT) and the other was attached to a fixed support glass rod. Transducer signals were fed into an 8-channel PowerLab A/D converter and recorded on a PC using Chart software (v. 3.4.9; iWorx, Dover, NH). After 10 min without tension, rings were equilibrated to ~1 g loading
tension over 30 min. Aortic rings were stimulated with 100 mM potassium-PSS (100 mM K+) to test for viability, then washed 3 times with PSS over 30 min, re-equilibrated to 1 g resting tension, and re-stimulated with 100 mM K+ followed by 3 bath changes and re-equilibration to 1 g. Rings from in vivo CS or acrolein exposures were contracted with cumulative concentrations of phenylephrine (PE; 0.1 nM - 1 µM) and then relaxed with cumulative concentrations of acetylcholine (ACh; 0.1 nM - 1 µM) to determine endothelium-dependent relaxation. Following a tension plateau, sodium nitroprusside (SNP; 100 µM) was added to determine endothelium-independent relaxation. Vessel contractions were quantified as raw (mg tension) or normalized as a percentage of the maximum PE contraction (in vivo exposure) or of the pre-treatment PE contraction (i.e., post-acrolein PE tension/pre-acrolein PE-tension %; in vitro exposure). Relaxation was calculated as the percentage reduction of PE-induced tension. The effective concentration producing 50 % response (EC50) was determined by normalizing cumulative concentration responses to 100 %, plotting response versus log [molar]agonist, and interpolating the EC50.

**Western Blotting:** SDS-PAGE and Western blotting were performed in aorta and lung homogenates (100 µg) and plasma (1 µl) with mouse monoclonal antibody against human GSTP1 (Invitrogen; 1:2,500) or IgG-purified rabbit anti-KLH-acrolein polyclonal antibody (1:1,000). Western blotting was also performed using whole lung homogenates for ALDH3A1, FR-1, HO-1, and NQO1 using in-house raised polyclonal or commercially available mouse monoclonal primary antibodies (1:1,000; HO-1, 9
Stressgen). Additionally, hepatic microsomes were probed for NQO1 and cytochromes P450 (1A1/1A2, 2B1, 2E1) using polyclonal in-house raised antibodies (1:1000). Western blots were developed using appropriate secondary antibodies and ECL® plus reagent (Amersham Biosciences), and band intensity detected with a Typhoon 9400 variable mode imager (Amersham Biosciences). Quantification of band intensity was performed using Image Quant TL software (Amersham Biosciences) and bands were normalized to actin or amido black staining. Relative protein level was calculated as a percentage of the appropriate air-exposed group.

**GST Activity and Immunohistochemistry:** Total GST conjugating activity toward a general substrate, 1-chloro,2,4-dinitrobenzene (CDNB; 1 mM), and a GSTP-selective substrate, ethacrynic acid (EA; 200 µM), was determined in lung and aorta homogenates according to Habig et al., (1974)(21). Immunohistochemistry was performed on formalin-fixed, paraffin-embedded tissue sections (4 µm) stained with rabbit polyclonal primary antibody against human GSTP1 (1:1,500; Novo Castra)(IgG-purified preimmune rabbit serum served as negative control)(19). Secondary antibody was an anti-rabbit goat antibody with a Vector Elite staining kit using diaminobenzadine (DAB; Dako) as chromagen.

**Statistical analysis:** Data are reported as mean ± SEM. For comparing two groups an unpaired Student’s t-test or Mann-Whitney Rank Sum test was used. Comparisons among multiple groups or between two groups at multiple time-points were performed
Results

**GSTP localization and abundance:** The GST gene superfamily comprises several members that show tissue-specific distribution (2; 47). To examine whether GSTP was expressed in lung and aorta, immunohistochemistry and Western analysis was performed. High levels of GSTP protein were detected in mouse aorta and lung (Fig. 1). In the aorta, positive GSTP staining was associated with areas also stained by the anti-von Willebrand factor (vWF; data not shown), although intense staining was also associated with the smooth muscle cells of the media (Fig. 1A). No staining was detected in vessels from GSP-null mice (Fig. 1A). Positive staining for GSTP protein was also observed in the WT lung where it was highly localized to lung airway columnar epithelium (Fig. 1A). GSTP protein was variably expressed in several other organs as well. Highest levels of GSTP were expressed in liver, aorta, stomach, and bladder (Fig. 1B).

To determine the contribution of GSTP to the total GST activity, GST activity was measured in lysates prepared from mouse lung and aorta (pooled from 3 mice). Total GST activity, measured using CDNB, in the lung was 27% of that obtained with the liver. Deletion of GSTP led to a <15% decrease in CDNB activity, suggesting that GSTP accounts for only a fraction of the total GST activity in the lung. In contrast, WT aortic GST activity was twice that present in the lung and was ~10 times that in aorta of
GSTP-null mice (Fig. 1C). Collectively, these results show GSTP is expressed in the lung and the aorta and although GSTP contributes to a small, but significant, fraction of the total tissue GST activity in lung, it comprises a majority of the GST activity in the murine aorta.

**Tobacco smoke toxicity:** To understand the role of GSTP in tobacco smoke toxicity, mice were exposed to a mixture of direct and side-stream smoke for 3 days and plasmatic changes were measured to assess systemic toxicity. As shown in Table 1, no increase in liver or muscle enzymes was observed, indicating that the exposure protocol did not induce overt toxicity. A decrease in total cholesterol was observed which was due to a small decrease in HDL. There was also a slight decrease in blood glucose in the tobacco smoke-exposed mice. The GSTP-null mice showed no changes in glucose and cholesterol levels. The levels of HDL and LDL in tobacco smoke-exposed GSTP-null mice were similar to air-exposed mice, which indicate that deletion of GSTP gene attenuates tobacco smoke-induced changes in blood glucose and cholesterol (Table 1). Even though no change in total protein was observed with WT mice, it was significantly decreased in GSTP-null mice.

**Tobacco smoke-induced endothelial dysfunction:** To examine how GSTP affects the vascular toxicity of tobacco smoke, mice were exposed for 3 days to tobacco smoke. The aorta was removed and its responses were studied *ex vivo*. As shown in Fig. 2, tobacco smoke exposure for 3 days did not significantly affect PE-induced tension...
development in WT mice. No significant change in ACh-induced relaxation was observed. In contrast, aortic rings prepared from similarly-exposed GSTP-null mice showed a much smaller extent of ACh-induced relaxation than the air-exposed mice (Fig. 2D). In aorta isolated from mice exposed to tobacco smoke, maximal relaxation due to ACh was nearly 60% of that observed in aorta of mice that inhaled air alone (Table 2). The GSTP-null mice also showed higher levels of PE-mediated contractility when compared with WT mice. Moreover, even though tobacco smoke-exposure did not affect SNP-mediated relaxation in WT mice; it led to a minimal, but statistically significant, decrease in SNP-induced relaxation in GSTP-null aorta (Table 2). The responses of aorta from naïve WT and GSTP-null mice to ACh, nitroglycerin (GTN), and SNP were similar; indicating that there were no basal differences in the aortic sensitivity between these two strains of mice (Table 2). Taken together, these data indicate that deletion of GSTP increases endothelial dysfunction induced by tobacco smoke.

Tobacco smoke and antioxidant defenses: Because tobacco smoke is known to induce oxidative stress, we tested whether induction of antioxidant enzymes in lung and liver were similar in WT and GSTP-null mice. After 3-day a tobacco smoke exposure, changes in lung and liver antioxidant enzymes were quantified by Western blotting (Table 3). No change was observed in lung or hepatic expression of NQO1, a Nrf2-dependent gene, however, a significant depression of hepatic NQO1 expression occurred in smoke-exposed GSTP-null mice compared with air-exposed GSTP-null controls (Fig. 3A). Pulmonary expression of FR-1, an aldo-keto reductase involved in
aldehyde metabolism (38), was increased in WT mice after 3-day tobacco smoke exposure (Fig. 3B), but this protein was down-regulated in lungs of GSTP-null mice after 3-day tobacco smoke exposure (Fig 3B). Hepatic microsomal CYP1A1 expression was significantly increased in tobacco smoke-exposed WT mice (Fig. 3C), however, CYP1A1 expression was significantly greater in air-exposed GSTP-null mice compared with WT air-exposed group, indicating a higher basal level of CYP1A1 expression in null mice that was neither enhanced nor suppressed by tobacco smoke exposure in GSTP-null mice (Fig. 3C). No change in the protein expression of other aldehyde-metabolizing enzymes, including ALDH3A1, GSTP, CYP2B1, or CYP2E1 was observed with tobacco smoke exposure (Table 3). After a 3-day tobacco smoke exposure, there was an ~10 % increase in total GST and GSTP activities, as well as, increased immunohistochemical staining for GSTP localized in airway epithelium (data not shown) in WT lung, but there was no change in total GST activity in GSTP-null lung compared with air-exposed GSTP-null lung (Fig. 3D). Moreover, GSTP activity measured with EA was ~2 % of the overall GST activity, yet was completely absent in the lung of air- and tobacco-smoke exposed GSTP-null mice (Fig. 3D). These data indicate that the tobacco smoke exposure used in the present study was sufficient to induce mild oxidative stress and the responses elicited by tobacco smoke exposure in the lung and the liver were dependent on the presence of GSTP.

Protein-acrolein adducts of CS exposure: Given that acrolein is one of the most toxic components of cigarette smoke (7) and a high affinity substrate of GSTP (8), we next
tested whether exposure to tobacco smoke leads to systemic delivery of acrolein from the lung to the peripheral tissues. As shown in Fig. 4, exposure to tobacco smoke led to an increase in the abundance of protein adducts of acrolein in the lung, the plasma and the aorta of tobacco smoke-treated WT mice compared with air-exposed controls immediately after 5h exposure to tobacco smoke as measured by Western blot analysis. Although low levels of protein-acrolein adducts, presumably due to basal oxidative stress, were observed in air-exposed lungs, significant increases were observed in the intensity of acrolein-adducted proteins of molecular weights 22, 30, 75, and 250 kDa in the lung of tobacco smoke-exposed lungs. A similar increase in the 150 kDa protein band also was observed in the plasma and the aorta (Fig. 4BCD). Additional proteins of different molecular weights were also modified by acrolein in the aorta, and a few protein bands were selectively increased in WT or GSTP-null mice exposed to tobacco smoke. For example, the protein band at ~150 kDa was significantly increased (P=0.003) in GSTP-null aorta but not WT aorta, while the 250 and 22 kDa bands were significantly increased in WT but not GSTP-null aorta exposed to tobacco smoke (Fig. 4CD). There was less immunohistochemical protein-acrolein adduct staining present in formalin-fixed tissues of 3-day tobacco smoke-exposed mice sacrificed 24h after the final exposure compared with tissues taken immediately after a 5h tobacco smoke exposure, indicating that protein-adducts of acrolein were rapidly removed or repaired (data not shown). Taken together, these data support the notion that tobacco smoke entering the lung delivers acrolein to the plasma and the aorta (and liver) and that deletion of GSTP while not grossly altering systemic delivery of acrolein, did affect the
aorta-specific generation of protein adducts of acrolein.

**Acrolein-induced endothelial dysfunction:** Given our results showing that acrolein was delivered to vascular sites in tobacco smoke-exposed mice, we tested whether GSTP regulates the endothelial and systemic toxicity of acrolein (Tables 4 & 5). For this, both WT and GSTP-null mice were exposed to inhaled acrolein. After exposure, aortas from acrolein- and air-exposed mice were removed and their sensitivity to ACh was examined *ex vivo*. As shown in Fig. 5, 3-day acrolein inhalation protocol (1 ppm; 5 h/day) had no effect on PE sensitivity of isolated aorta of WT or null mice (Fig. 5AB). However, acrolein decreased ACh-induced relaxation in GSTP-null, but not in WT mice (Fig. 5CD); indicating that deletion of GSTP gene exacerbates acrolein-induced endothelial dysfunction (Table 4).

**Protein-acrolein adducts of acrolein exposure:** Because protein-acrolein adducts were present in peripheral tissues after tobacco smoke exposure, we tested whether direct exposure to acrolein would also lead to a similar tissue distribution and accumulation of protein-acrolein adducts. As shown in Fig. 6, acrolein exposure led to an increase in the abundance of acrolein-protein adducts in the lung, the plasma and the aorta of acrolein-treated mice compared with air-exposed controls immediately after 5h exposure as measured by Western blot analysis. Although low levels of protein-acrolein adducts were observed in air controls (as in Fig. 4), significant increases in the intensity of acrolein-adducted proteins of molecular weights ~22, ~30, ~75, and ~250 kDa were
observed in the lung. A similar increase in a ~150 kDa protein band also was observed in the plasma and the aorta (Fig. 6BCD). Additional proteins of different molecular weights were also modified by acrolein in the aorta with a few protein bands selectively increased in GSTP-null mice exposed to acrolein. For example, bands of ~150 (P=0.055), ~75 (P=0.029) and ~70 (P=0.002) kDa were significantly increased in GSTP-null aorta but not in WT aorta, while the 250 kDa band was also significantly increased in GSTP-null aorta exposed to acrolein, but to a lesser degree in WT mice (Fig. 6CD). There was less immunohistochemical protein-acrolein adduct staining present in formalin-fixed tissues of 3-day acrolein-exposed mice sacrificed 16h after the final exposure compared with tissues taken immediately after a 5h acrolein exposure, indicating that protein-adducts of acrolein were rapidly removed or repaired (data not shown). Taken together, these data confirm that tobacco smoke entering the lung delivers acrolein to the plasma and the aorta and that deletion of GSTP, while not altering systemic delivery of acrolein, increases the formation of specific acrolein-protein adducts in the aorta.

Finally, to examine the role of GSTP in directly modulating acrolein toxicity, aortic rings prepared from naïve mice were treated with acrolein in the tissue bath. As shown in Fig. 7A, treatment with acrolein led to a concentration-dependent decrease in ACh-induced relaxation of precontracted aortic rings prepared from C57BL/6 mice in vitro. Although ACh-induced relaxations in control (untreated) aorta of WT and GSTP-null mice were similar, the extent of acrolein-induced inhibition was much greater in GSTP-null mice than in WT mice (Fig. 7B). The exaggerated endothelial dysfunction in GSTP-
null mice induced by acrolein was rescued by pre-treatment with NAC (Fig. 7B). NAC treatment alone did not affect aortic reactivity. Collectively, these results suggest that GSTP protects against endothelial dysfunction induced by direct acrolein exposure. That the high sensitivity of GSTP-null mice to acrolein could be prevented by NAC pretreatment supports the notion that the toxicity of acrolein could be attenuated by thiol conjugation.

**Discussion**

The major findings of this study are that deletion of the GSTP gene exacerbates endothelial dysfunction induced by exposure to tobacco smoke or to inhaled acrolein. We hypothesized that smoking-induced endothelial dysfunctions was mediated in part by electrophilic constituents of cigarette smoke such as acrolein. Hence, detoxification of electrophilic cigarette smoke constituents by GSTP may be a protective mechanism against the vascular effects of tobacco smoke and other acrolein-rich pollutants (e.g., coal smoke, wood smoke, automobile exhausts). The results obtained support the hypothesis and significantly advance our understanding of the vascular toxicity of tobacco smoke and the processes that modulate the CVD risk of smoking.

The endothelium appears to be a highly vulnerable target of tobacco smoke. Smoking injures endothelial cells and vessels isolated from chronic smokers show degenerative changes (10). In humans, smoking diminishes flow-mediated endothelium-dependent vasodilation (5) and serum from smokers decreases NO
production and endothelial nitric oxide synthase (eNOS) activity in endothelial cells (6). Moreover, aorta isolated from tobacco smoke-exposed animals shows impaired endothelium-dependent relaxation (36). In agreement with these results, we found that a short, 3-day exposure to tobacco smoke diminished acetylcholine-induced relaxation in aorta isolated from exposed GSTP-/- mice. This early and significant dysfunction underscores the high vulnerability of the endothelium to tobacco smoke exposure. Significantly, similar inhibition of acetylcholine-mediated relaxation was observed when the GSTP-/- mice were exposed to acrolein, which is one of the most reactive and toxic chemicals in cigarette smoke. Taken together, these observations raise the possibility that endothelial dysfunction induced by tobacco smoke could be in part attributable to acrolein and related electrophilic components of cigarette smoke.

A significant role of acrolein and related electrophiles in tobacco smoke toxicity is also consistent with the observation that deletion of GSTP, which catalyzes the conjugation of acrolein with glutathione with 6- to 35-fold higher efficiency than other GSTs (8), increased both acrolein- and tobacco smoke-induced endothelial dysfunction. Endothelium-dependent effects of acrolein were abrogated by pretreatment with N-acetylcysteine (NAC) consistent with the ability of thiol conjugation to mitigate unsaturated aldehyde-induced injury. Thus, the observations that both chemical (NAC) and enzymatic (GSTP) quenching of acrolein, an electrophilic constituent of cigarette smoke, were effective in preventing the vascular toxicity of tobacco smoke support the idea that thiol-reactive components of cigarette smoke (of which acrolein is the most
reactive) are significant mediators of cigarette smoke-induced endothelial dysfunction.

Acrolein and related aldehydes are present in high concentration in cigarette smoke. Several reports estimate that between 100-600 μg acrolein are generated per cigarette (50 to 70 ppm) and that acrolein constitutes 50-60 % of the total vapor phase electrophiles (15; 18; 39). Because most of acrolein is generated during smoldering, its concentration in sidestream smoke is 10- to 12-fold higher than in mainstream smoke (18). This could explain, in part, the finding that even though the dose of smoke delivered to active smokers is approximately 100-times more than that delivered to passive smokers, the relative rate of CVD for smokers is 1.78 compared with 1.31 for passive smokers (4). Hence, decreasing acrolein emissions and exposures or increasing acrolein metabolism via GSTP may help in preventing the cardiovascular toxicity of tobacco smoke.

Our results show that exposure to tobacco smoke results in the appearance of protein-acrolein adducts in the lung, plasma and in the aorta of exposed animals. These observations suggest that despite its high reactivity acrolein is delivered from the lung into systemic circulation and to vascular sites. Smokers’ urine contains high concentrations of acrolein metabolites (6-8 μM) demonstrating presence of systemic acrolein and contribution of acrolein metabolism (12). However, it is not clear whether these metabolites are generated in the lung and then excreted in the urine or whether they are derived from non-pulmonary metabolism, including hepatic. Hence our observation that tobacco smoke (and acrolein) exposure led to the formation of specific
and shared protein-acrolein adducts in the plasma and the aorta suggest that free acrolein is transported from the lung into the blood, and thus, could cause direct vascular toxicity. Although current data cannot rule out the possibility that glutathione conjugates formed in the lung dissociate in the blood, increased formation of adducts at non-pulmonary sites support the concept that non-pulmonary metabolism of acrolein by enzymes such as GSTP may be an important determinant of vascular toxicity due to cigarette smoke. This view is consistent with our observation that deletion of GSTP led to an increase in the abundance of specific acrolein protein adducts in the aorta. These observations indicate that the extent of acrolein reactivity in the aorta may be, in part, regulated by the metabolic capacity intrinsic to aorta.

Exacerbation of tobacco smoke-induced protein-acrolein adducts accumulation and endothelial dysfunction in GSTP-null mice indicates that this enzyme may be an important modulator of CVD risk due to smoking in humans. Although complete GSTP deficiency is rare in humans, several known polymorphisms of the human GSTP gene have been identified. Of these, the hGSTP1 (I104,A113) allele is the most frequent in human populations, however, the frequency of hGSTP (V104, A113) and/or hGSTP (V104, V113) allele is higher in certain cancers (22; 45). Previous studies show that the catalytic efficiency of GSTPVV form with acrolein is much less than that of other polymorphic variants of GSTP (130 versus 90 mM⁻¹s⁻¹)(35). The VV form also differs in catalytic efficiency with benzo[a]pyrene diol epoxide (26; 27). Hence, our observation that GSTP deficiency in mice increases the vascular toxicity of tobacco smoke and
acrolein in mice raises the possibility that humans with the VV isoform of GSTP may be at a higher risk of tobacco smoke-induced CVD than those who possess other allelic forms of GSTP. Epidemiology studies have associated GSTP polymorphisms with a variety of respiratory outcomes that may reflect functional changes in airway responsiveness to endogenous and exogenous electrophiles (3; 31; 32; 40). Nevertheless, the effects of GSTP polymorphism on cigarette smoke-induced endothelial dysfunction remain unknown. Although additional epidemiological studies are required to assess this risk, we have observed that human coronary artery bypass graft (CABG) blood vessels (arteries and veins) express GSTP in high abundance, which indicates that this enzyme may be an important determinant of the response of human vessels to tobacco smoke as well (DJ Conklin, unpublished data). Furthermore, in addition to differences in polymorphic alleles, individual differences in GSTP activity may arise also from enzyme induction. GSTP is a highly inducible enzyme. Although GSTP was not induced by tobacco smoke in our model, the gene is induced by a variety of environmental and dietary factors such as the garlic organosulfur compounds (44), chemopreventive selenocysteine conjugates (1) and coffee (42). Hence, GSTP induction by such agents could modify the CVD risk of cigarette smoking.

It is currently believed that induction of GSTP protects tumors from anti-cancer therapy via conjugation and removal of electrophilic products or by preventing apoptosis (28). In our present study, we did not observe significant induction of lung GSTP, but we did see tobacco smoke-induced upregulation of other antioxidant proteins in a
GSTP-dependent manner. As shown in Figure 3, tobacco smoke exposure significantly increases AKR1B8 (FR-1) protein in WT mice and tobacco smoke increases pulmonary expression of FR-1 mRNA (37). FR-1 is an antioxidant protein involved primarily in reduction of lipid peroxidation aldehydes, such as HNE, and is a mouse homolog of aldose reductase (41). Thus, it is surprising that pulmonary FR-1 protein expression under regulatory control of Nrf2 was significantly down-regulated in GSTP-null mice after tobacco smoke exposure, because this would likely lead to a decrease in the overall aldehyde detoxification capacity. Because FR-1 reduces a wide array of aldehydes, a decrease in FR-1 levels, coupled with GSTP-deficiency, could significantly elevate the levels of free acrolein and other secondary aldehydes generated by cigarette smoke-induced oxidative stress in the lung, and thus, increase the overall toxicity of tobacco smoke. In addition, GSTP also seems to protect against the systemic toxicity of tobacco smoke. Our observations that deletion of the murine GSTP prevented tobacco smoke-induced decreases in HDL, triglycerides, and blood glucose (Table 1) and the induction of CYP1A1/1A2 (Table 3) suggest that adaptive changes to tobacco smoke are blunted in GSTP-null mice. This surprising effect of GSTP deficiency on the transcriptional regulation of other antioxidant enzymes and the induction of adaptive responses has not been described before, and could provide an important clue for understanding how GSTP polymorphisms may contribute to increased cardiopulmonary sensitivity to xenobiotic exposures in humans.

In addition to tobacco smoke, acrolein and related aldehydes are also a
component of automobile exhaust, smog, cotton, wood, and coal smoke as well as the ambient air (9). The concentration of acrolein in ambient air ranges from 0.003 to 0.01 ppm, whereas 0.04 to 2.2 ppm has been detected near automobile exhaust (18).

Acrolein is also endogenously generated during inflammation and lipid peroxidation (9). High (0.7 to 2 μM) concentrations of S-(3-hydroxypropyl)mercapturic acid, the major metabolite of acrolein, have been detected in the urine of healthy young adults and the concentration of the acrolein metabolite exceeds that of another lipid peroxidation product – 4-hydroxy-trans-2-nonenal (HNE) -- by a factor of 100 (43); suggesting that high levels of acrolein are generated endogenously. Hence, our observations that GSTP deficiency increases the vascular toxicity of inhaled acrolein and acrolein exposure *ex vivo* raise the possibility that GSTP may be a critical determinant of cardiovascular injury due to inflammation or exposure to several environmental pollutants other than tobacco smoke as well.
Acknowledgments

We thank S.O. Awe, B. Bishop, D. Bolanowski, D. Mosley, A. Tang, E. Werkman and D. Young for technical assistance. We thank Dr. S. Myers for use of the smoke machine. We thank Drs. C. Henderson and R. Wolf, University of Dundee, for providing breeding pairs of GSTP mice. We thank Dr. P.C. Burcham, University of Western Australia, for gift of anti-protein-acrolein antisera.

Grants

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Conflict of Interest

Disclosures: None
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Figure Legends

Figure 1. Tissue abundance of GSTP protein: (A) Lung and aortic distribution of GSTP protein in naïve, male WT and GSTP-null mice. Positive immunohistological staining was present in WT lung (most intense in the pseudostratified columnar epithelium nuclei; arrows) and aorta but was absent in GSTP-null tissues; (B) Western blots with anti-GSTP antibody using lysates obtained from aorta, urinary bladder, stomach, small intestine, lung, liver, kidney, and heart from WT (+) and GSTP-null (-) mice; and (C) Catalytic activity of GST in mouse lung and aorta. Total GST activity was measured using 1-chloro-2,4-dinitrobenzene (CDNB) in lung (WT=3; null=3) and whole aorta (WT=4; null=4) homogenates of WT and GSTP-null mice. * P<0.05.

Figure 2. GSTP-deficiency increased susceptibility to tobacco smoke-induced endothelial dysfunction. Aortas were removed from WT and GSTP-null mice exposed for 3 days to cigarette smoke as described in the text and their contractile and relaxant responses to cumulative concentrations of (A, C) phenylephrine (PE) and (B, D) acetylcholine (ACh) were measured, respectively (WT=4; null=4). Control mice (WT=4; null=3) were exposed to air alone. * P<0.05.
Figure 3. Cigarette smoke (CS) exposure (3-day) induced lung and liver antioxidant proteins. (A) Fibroblast growth factor regulated protein (FR-1) was induced in lung of WT mice and was down-regulated in GSTP-null mice. (B) Basal hepatic expression of NAD(P)H:quinone oxidoreductase (NQO1) was greater in GSTP-null mice compared to WT mice. CS exposure down-regulated hepatic NQO1 expression in GSTP-null mice. (C) Basal hepatic expression of cytochrome P450 1A1/1A2 (CYP 1A1) was greater in GSTP-null mice compared with WT mice. CS induced hepatic CYP1A1 expression in WT mice. (D) Catalytic activity of GST in mouse lung. Total GST and GSTP activities in lung homogenates of WT and GSTP-null mice exposed to air or CS were measured using 1-chloro-2,4-dinitrobenzene (CDNB) or ethacrynic acid (EA), respectively. Whole lung homogenate or hepatic microsomal proteins (25-100 µg) were blotted with polyclonal antibodies and band density normalized to total amido black staining. Protein expression was calculated as a percentage of WT air-exposed samples. * P<0.05, significant difference between bar with symbol and WT air-exposed mice; # P<0.05, significant difference between bar with symbol and GSTP-null air-exposed mice (n=3 mice/group).
**Figure 4.** Exposure to tobacco smoke results in systemic delivery of acrolein. Western blots showing protein-acrolein adducts in the (A) lung, (B) plasma and (C, D) aorta of mice exposed to air or tobacco smoke (CS). Adult male WT and GSTP-null mice were exposed for 5 h to tobacco smoke or air and euthanized immediately after exposure. Protein-acrolein adducts were detected in lysates of tissues frozen immediately after exposure using purified-IgG rabbit polyclonal anti-KLH-acrolein antibody. Summary data of major protein-acrolein adducts in tissues was normalized to total amido black-stained protein. * P < 0.05; † 0.10>P>0.05; (for A, C and D, n = 3 mice/group; for B, n=4 mice/group).

**Figure 5.** GSTP-dependent endothelial dysfunction of inhaled acrolein. WT and GSTP-null mice were exposed to air or acrolein (1 ppm) by inhalation for 3 days (5 h/day) and sacrificed 16 h after the final exposure. Isolated thoracic aorta (~5 mm) sensitivity to (A,B) phenylephrine (PE) and (C,D) acetylcholine (ACh) were measured to assess contractility and endothelium-dependent relaxation, respectively. * P<0.05; WT=4,4 mice/group; Null=8,8 mice/group.
**Figure 6.** Exposure to acrolein results in systemic delivery of acrolein. Western blots showing protein-acrolein adducts in the (A) lung, (B) plasma and (C, D) aorta of mice exposed to air or acrolein. Adult male WT and GSTP-null mice were exposed for 5 h to acrolein (5 ppm) or air and then euthanized immediately after exposure. Protein-acrolein adducts were detected in lysates of tissues frozen immediately after exposure using purified-IgG rabbit polyclonal anti-KLH-acrolein antibody. Summary data of major protein-acrolein adducts in tissues was normalized to total amido black-stained protein. * P < 0.05; † 0.10>P>0.05; (for A, C and D, n = 3 mice/group; for B, n=4 mice/group).

**Figure 7.** Acrolein toxicity in WT and GSTP-null mice. (A) Acetylcholine (ACh)-stimulated relaxation of phenylephrine-precontracted (PE) aorta of naïve male C57BL/6 mice treated with acrolein. Naïve aortic rings (n=3-5) were exposed to normal buffer (Control) or acrolein (10, 40 or 80 µM) for 90 min before measuring response to ACh in PE-precontracted aorta. (B) Effects of N-acetylcysteine (NAC) on ACh-induced relaxation in acrolein pretreated aorta of naïve WT and GSTP-null mice. * P<0.05 vs. GSTP-null control. † P<0.05 vs. acrolein treatment in WT mice (n=4 mice/group).
Table 1. Blood parameters in wild type (WT) and GSTP-null mice exposed to air or cigarette smoke (CS).

<table>
<thead>
<tr>
<th></th>
<th>WT</th>
<th>GSTP-null</th>
<th>WT</th>
<th>GSTP-null</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Air</td>
<td>CS</td>
<td>Air</td>
<td>CS</td>
</tr>
<tr>
<td>Blood Glucose</td>
<td>226±8</td>
<td>167±6 *</td>
<td>199±9</td>
<td>186±15</td>
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<td>HCr (%)</td>
<td>45.9±0.3</td>
<td>49.3±0.9*</td>
<td>47.9±0.6</td>
<td>47.8±1.4</td>
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<td>Buffy Coat (%)</td>
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<td>1.3±0.1</td>
<td>1.3±0.2</td>
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<td>Cholesterol</td>
<td>117±4</td>
<td>101±6 *</td>
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<td>90±2</td>
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<tr>
<td>HDL</td>
<td>88±3</td>
<td>69±5 *</td>
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</tr>
<tr>
<td>LDL</td>
<td>23±2</td>
<td>29±3</td>
<td>18±1</td>
<td>15±1</td>
</tr>
<tr>
<td>Triglycerides</td>
<td>38±5</td>
<td>20±4 *</td>
<td>28±3</td>
<td>27±3</td>
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<td>TP</td>
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<td>4.61±0.05*</td>
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<td>3.18±0.04</td>
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<td>2.94±0.04</td>
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<td>LDH</td>
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<td>202±17</td>
<td>165±16</td>
<td>154±13</td>
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<tr>
<td>CK</td>
<td>166±22</td>
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<td>164±11</td>
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<tr>
<td>AST</td>
<td>48±6</td>
<td>62±6</td>
<td>56±2</td>
<td>62±6</td>
</tr>
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</table>

Male, 12-14 week old WT or GSTP-null mice were exposed to air or CS (5h/day) for 3 days. Mice were euthanized 24h after exposure following an 8h fast. Units: \( ^a = \text{[mg/dl]} \), \( ^b = \text{[g/L]} \), \( ^c = \text{[U/L]} \), \( ^d = \text{[ng/ml]} \); values are in mean±SEM; * P<0.05 vs. air-matched group; n=7-8 mice/group. Abbr.: HCr, hematocrit; HDL, high density lipoprotein cholesterol; LDL, low density lipoprotein cholesterol; TP, total protein; ALB, albumin; LDH, lactate dehydrogenase; CK, creatine kinase; ALT, alanine aminotransferase; AST, aspartate aminotransferase.
Table 2. Vascular effects of air or cigarette smoke (CS) exposure in wild-type (WT) and GSTP-null mice.

<table>
<thead>
<tr>
<th></th>
<th>WT</th>
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<tr>
<td></td>
<td>Air</td>
<td>CS</td>
<td>Air</td>
<td>CS</td>
</tr>
<tr>
<td>Phenylephrine (PE)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tension (mg)</td>
<td>1256±196</td>
<td>1112±119</td>
<td>875±203</td>
<td>1246±214</td>
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<tr>
<td>Tension (g/mg wet wt)</td>
<td>1.16±0.19</td>
<td>0.88±0.13</td>
<td>0.57±0.13</td>
<td>1.07±0.21</td>
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<tr>
<td>Tension (PE/HI K⁺)</td>
<td>106±3</td>
<td>93±10</td>
<td>107±4</td>
<td>102±12</td>
</tr>
<tr>
<td>EC₅₀ (nM)</td>
<td>68±17</td>
<td>94±19</td>
<td>83±24</td>
<td>107±33</td>
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<tr>
<td>pD₂</td>
<td>7.18±1.61</td>
<td>7.07±0.12</td>
<td>7.11±1.59</td>
<td>7.01±1.57</td>
</tr>
<tr>
<td>Acetylcholine (ACh)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Relaxation (% PE)</td>
<td>-61±68</td>
<td>-73±10</td>
<td>-80±9</td>
<td>-48±9*</td>
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<tr>
<td>EC₅₀ (nM)</td>
<td>328±115</td>
<td>665±454</td>
<td>132±54</td>
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<td>pD₂</td>
<td>6.55±1.47</td>
<td>6.52±0.32</td>
<td>6.71±1.52</td>
<td>6.76±1.52</td>
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<tr>
<td>Glyceryl Trinitrate (GTN)†</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Relaxation (% PE)</td>
<td>-114±17</td>
<td>ND</td>
<td>-103±4</td>
<td>ND</td>
</tr>
<tr>
<td>EC₅₀ (nM)</td>
<td>27±7</td>
<td>ND</td>
<td>83±67</td>
<td>ND</td>
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<tr>
<td>pD₂</td>
<td>7.63±0.10</td>
<td>ND</td>
<td>7.60±0.32</td>
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<tr>
<td>Sodium Nitroprusside (SNP)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Relaxation (% PE)</td>
<td>-110±8</td>
<td>-105±17</td>
<td>-158±20</td>
<td>-100±6*</td>
</tr>
<tr>
<td>EC₅₀ ‡ (nM)</td>
<td>38±21</td>
<td>ND</td>
<td>9±3</td>
<td>ND</td>
</tr>
<tr>
<td>pD₂ ‡</td>
<td>7.68±0.19</td>
<td>ND</td>
<td>8.12±0.13</td>
<td>ND</td>
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</tbody>
</table>

Male, 12-14 week old WT or GSTP-null mice were exposed to air or CS (5h/day) for 3 days. Mice were euthanized 24h after exposure following an 8h fast. Abbr: HI K⁺, 80 mM potassium buffer; EC₅₀ = effective concentration producing 50% response; pD₂, -log[EC₅₀]; ND, not determined; * P< 0.05 vs. matched air-exposed control (n=4 mice/group); †, data from naïve mice only (n=4 mice/group).
Table 3. Expression of antioxidant enzymes in lung and liver after 3-day air or cigarette smoke (CS) exposure in male wild-type (WT) and GSTP-null mice.

Western blot band intensity was normalized to total lane amido black staining. Relative protein expression was calculated as a percentage of WT air-exposed value. * P<0.05 significance between group with symbol and WT air-exposed group (n=3 mice/group).
Table 4. Vascular effects of air or acrolein in wild type (WT) and GSTP-null mice.

<table>
<thead>
<tr>
<th></th>
<th>WT</th>
<th>GSTP-null</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Air</td>
<td>Acrolein</td>
</tr>
<tr>
<td>Phenylephrine (PE)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tension (mg)</td>
<td>792±140</td>
<td>1030±130</td>
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<tr>
<td>Tension (g/mg wet wt)</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>Tension (PE/100 mM K⁺)</td>
<td>116±17</td>
<td>139±28</td>
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<tr>
<td>EC₅₀ (nM)</td>
<td>125±15</td>
<td>120±18</td>
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<tr>
<td>pD₂</td>
<td>6.91±0.05</td>
<td>6.94±0.06</td>
</tr>
<tr>
<td>Acetylcholine (ACh)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Relaxation (% PE)</td>
<td>-66±7</td>
<td>-76±13</td>
</tr>
<tr>
<td>EC₅₀ (nM)</td>
<td>119±29</td>
<td>138±50</td>
</tr>
<tr>
<td>pD₂</td>
<td>6.98±0.13</td>
<td>6.97±0.19</td>
</tr>
<tr>
<td>Sodium Nitroprusside (SNP)</td>
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<tr>
<td>Relaxation (% PE)</td>
<td>-118±28</td>
<td>-152±46</td>
</tr>
</tbody>
</table>

Male, 12-14 week old WT or GSTP-null mice were exposed to air or acrolein (1ppm, 5h/day) for 3 days. Mice were euthanized 16h after final exposure. Abbr: HI K⁺, 100 mM potassium buffer; EC₅₀ = effective concentration producing 50% response; pD₂, -log[EC₅₀]; ND, not determined; * P<0.05 vs. matched air-Control (n=4-8 mice/group).
Table 5. Blood parameters in wild type (WT) and GSTP-null mice exposed to air or acrolein.

<table>
<thead>
<tr>
<th></th>
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<th>Acrolein</th>
<th>GSTP-null</th>
<th>Acrolein</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Air</td>
<td>Acrolein</td>
<td>Air</td>
<td>Acrolein</td>
</tr>
<tr>
<td>Hct (%)</td>
<td>45.0±0.5</td>
<td>46.0±1.0</td>
<td>41.3±0.8</td>
<td>44.7±0.5</td>
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<tr>
<td>Buffy Coat (%)</td>
<td>0.9±0.0</td>
<td>1.7±0.0 *</td>
<td>1.5±0.1</td>
<td>1.4±0.1</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>94±2</td>
<td>95±3</td>
<td>113±3</td>
<td>112±2</td>
</tr>
<tr>
<td>HDL a</td>
<td>68±3</td>
<td>64±2</td>
<td>75±3</td>
<td>77±2</td>
</tr>
<tr>
<td>LDL a</td>
<td>14±1</td>
<td>16±1</td>
<td>15±1</td>
<td>13±1</td>
</tr>
<tr>
<td>Triglycerides a</td>
<td>76±8</td>
<td>72±5</td>
<td>76±7</td>
<td>56±4*</td>
</tr>
<tr>
<td>TP b</td>
<td>4.78±0.08</td>
<td>4.83±0.11</td>
<td>4.73±0.08</td>
<td>4.70±0.13</td>
</tr>
<tr>
<td>ALB b</td>
<td>2.96±0.03</td>
<td>3.04±0.02</td>
<td>2.88±0.07</td>
<td>3.04±0.08</td>
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<tr>
<td>LDH c</td>
<td>140±12</td>
<td>160±1</td>
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<td>235±24</td>
</tr>
<tr>
<td>CK c</td>
<td>209±28</td>
<td>275±26</td>
<td>157±12</td>
<td>156±13</td>
</tr>
<tr>
<td>ALT c</td>
<td>28±1</td>
<td>28±1</td>
<td>34±2</td>
<td>31±2</td>
</tr>
<tr>
<td>AST c</td>
<td>59±6</td>
<td>69±4</td>
<td>64±4</td>
<td>66±5</td>
</tr>
</tbody>
</table>

Male, 12-14 week old WT or GSTP-null mice were exposed to air or acrolein (1ppm; 5h/day) for 3 days. Mice were euthanized 16h after exposure. Units: a = [mg/dl], b = [g/L], c = [U/L]; values are in mean±SEM; * P<0.05 vs. matched air-Control group; WT=4 and GSTP-null=8 mice/group. Abbr.: Hct, hematocrit; HDL, high density lipoprotein cholesterol; LDL, low density lipoprotein cholesterol; TP, total protein; ALB, albumin; LDH, lactate dehydrogenase; CK, creatine kinase; ALT, alanine aminotransferase; AST, aspartate aminotransferase.
Fig. 1.

**A.**

Lung Aorta

WT

Null

**B.**

25 kDa

- Aorta + Bladder + Stomach + Small + Lung + Liver + Kidney + Heart

**C.**

GST ACTIVITY (nmol CDNB/min/mg protein)

- WT LUNG - NULL LUNG - WT AORTA - NULL AORTA
Fig. 2.

A.

B.

C.

D.
**Fig. 3.**

A. Bar graph showing NQO1 expression levels (Normalized band density, % WT) for GSTP WT and GSTP-null groups under different treatment conditions (WT AIR, WT CS, NULL AIR, NULL CS).

B. Bar graph showing FR-1 expression levels (Normalized band density, % WT) for GSTP WT and GSTP-null groups under different treatment conditions (AIR, CS).

C. Bar graph showing CYP1A1 expression levels (Normalized band density, % WT) for GSTP WT and GSTP-null groups under different treatment conditions (WT AIR, WT CS, NULL AIR, NULL CS).

D. Bar graph showing lung GST activity (% WT AIR) under different treatment conditions (WT AIR, WT CS, NULL AIR, NULL CS), with GST substrates CDNB and EA.
Fig. 4.
Fig. 5.

A. B.

C. D.
Fig. 6.

A. LUNG

B. PLASMA

C. AORTA

D. AORTA

**Protein-Acrolein Adducts**

- WT
- Null
A.

B.

Fig. 7.