Supplemental Material

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Running Title: *I/R injury in GSTP-null mice*

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Key Words: acrolein, aldehydes, glutathione *S*-transferase, glutathiolation, myocardial infarction, lipid peroxidation, protein adduction, oxidative stress

Non-standard Abbreviations: AR, aldose reductase; DMPO, dimethyl propyloxide; GSH, reduced glutathione; GST, glutathione *S*-transferase; 4HNE, 4-hydroxy-*trans*-2-nonenal; I/R, ischemia-reperfusion; JNK, c-jun N-terminal kinase; MPO, myeloperoxidase; MRM, multiple reaction monitoring; QDA, N-[2-(aminooxy)ethyl]-N,N-dimethyl-1-dodecylammonium iodide

Supplemental Methods:

Mice. Glutathione *S*-transferase-P1/P2 WT and null mice were generated on a MF1 background strain using homologous recombination as reported.[1] Mice heterozygous for the targeted locus (F1) were backcrossed and $GSTP^{(-/-)}$, $GSTP^{(+/-)}$ and $GSTP^{(+/+)}$ lines were established. GSTP-null and GSTP wild type (WT) littermates were obtained from Drs. C. Henderson and R. Wolf (Univ. Dundee), crossed to 10 generations on C57BL/6J mice and maintained as independent lines for use in this study. Mice were treated in accordance with the Declaration of Helsinki and with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the U.S. National Institutes of Health. Protocols were approved by the University of Louisville IACUC.

PCR Protocol for GSTP1-1/P2-2 Screening: PCR products were used to genotype WT and null mice using primers that amplified a region between exons 5 and 6 of GSTP1 and a region in the lacZ gene to identify a null allele. Primers [5' – 3'] were: WT (P1 ggccacccaactactgtgat; P2 agaaggccaggtcctaaagc) and null (P3 ctgtagcggctgatgttgaa; P4 atggcgattaccgttgatgt). All four primers were mixed with tail DNA, amplified using *Taq* polymerase (Promega, Madison, WI), and products run on 2% agarose gel with WT band at 200 bp and null band at 300 bp.

GST Expression and Abundance. Real time-PCR and Western blotting for tissue expression of GSTs (A, M, and P) was performed using commercially available standards and antibodies. For GSTP, a mouse monoclonal antibody against human GSTP1 (Invitrogen, 1:2,500) was used. Alpha-tubulin or amido black staining was used as loading control. Quantification of band intensities was performed using Image Quant TL software (Amersham Biosciences).

Total RNA Isolation: Approximately 30 mg of pulverized heart tissue was used to isolate total RNA using Trizol (Invitrogen) and RNeasy MiniKit according to manufacturer's instructions. The total RNA was eluted from miRNeasy minicolumns in 30 µL of RNAse free water. RNA concentration was determined using NANODROP 2000C (Thermo Scientific). Total cDNA was synthesized using iScript cDNA synthesis Kit from BIO-RAD (#170-8891) according to manufacturer's instructions. Briefly, 15 µL denatured RNA (1 µg), 4 µL 5X iScript

reaction mix and 1 μL of iScript reverse transcriptase were mixed (total volume, 20 μL) and cDNA synthesis was carried out in a Bio-Rad mycycler Thermocycler using the following conditions: 42^0C for 60 min and 94^0C for 5 min. If not used right away, the cDNA samples were stored at -20^0C. Total cDNA were diluted 20 fold using RNAse free water just before use. Real-time quantitative PCR was done using iTaq Universal SYBR green supermix from BIO-RAD (#172-5121) according to the manufacturer's instructions, Briefly, the following were added to each well of a 384-well plate sequentially: 5.0 μL iTaq 2X Universal SYBR green supermix, 2 μL diluted cDNA and 3 μL of mouse GST isoform specific (**Online Table I**) or mGAPDH (internal control) primers (for a total of 10 μL final volume) and subjected to qRT-PCR using standard protocols on the Applied Biosystems 7900 HT Real-Time PCR system. For each RNA sample, cDNAs were run in triplicate for each primer set in the same plate along with GAPDH. The RQ of GSTs for each RNA sample was determined by $2^{-\Delta\Delta CT}$, where ΔCT= (average of triplicate $CT_{\text{Target GST}}$ − average of triplicate $CT_{\text{endogenous control GAPDH}}$) and $\Delta\Delta CT$= (average $\Delta CT_{\text{experimental GST}}$−average $\Delta CT_{\text{vehicle GST}}$).

GST Enzymatic Activity: Total GST conjugating activity toward substrates 1-chloro-2,4-dinitrobenzene (CDNB; 1 mM) and ethacrynic acid (EA; 200 μM) was determined in cardiac homogenates according to Habig *et al*., (1974)[2] and as previously reported.[3, 4]

Ischemia/Reperfusion-Induced Cardiac Injury. Male WT and GSTP-null mice (12-16 weeks old) were used. Mice had free access to food and water in all treatments.

Echocardiography: Echocardiography was performed on Avertin-anesthetized male mice (12-16 weeks old; Philips Sonos 5500 Ultrasound System). In a subset of mice, a Millar pVR-1045 1 Fr catheter was inserted into LV via right carotid artery and aortic valve to record basal LV pressures.

Cardiac Abundance of Antioxidant Proteins and Activity, Glutathione and Markers of Oxidative Stress: To determine if basal oxidative stress was increased in hearts of GSTP-null mice, expression levels of protein and activities of several well-known antioxidant enzymes, including aldose reductase (AR), thioredoxin (Trx), γ-glutamatyl cysteine ligase (GCL; rate

limiting enzyme in GSH synthesis), and heme oxygenase-1 (HO-1; a Nrf-2 inducible protein), were measured by Western blotting in GSTP-null and WT mice using commercially-available or in-house antibodies. Activities of superoxide dismutase (SOD), catalase, and glutathione peroxidase (H_2O_2 and cumene hydroperoxide) were measured using standard techniques in heart homogenates. For measurement of cardiac levels of reduced (GSH) and oxidized (GSSG) glutathione, a recycling kit was used (Biooxytech GSH/GSSG-412, Oxis International, Inc., Foster City, CA, USA; sold by Percipio Biosciences). The hearts were powdered using liquid N_2 and a Bessman tissue pulverizer. Powders were divided into two aliquots (10 mg) that were homogenized in a water solution containing ice-cold 5% metaphosphoric acid (80 µl/mg tissue) with and without 1-methyl-2-vinyl-pyridiniumtrifluoromethane sulphonate (M2VP; 10% v/v), which binds with free GSH. The homogenates were then centrifuged at 16,000xg for 15 min at 4°C. For GSH, supernatant (4 µl) was mixed with 96 µl GSH assay buffer. For GSSG measurement, 5 µl of the supernatant (with M2VP) was mixed with 95 µl GSSG assay buffer. For both assays, samples were mixed with 300 µl of chromagen, glutathione reductase, and allowed to incubate for 5 min (RT). The assay reaction was then started with the addition of 300 µL NADPH, and the reduction of dithiobis-2-nitrobenzoic acid (DTNB) was monitored by measuring absorbance at 412 nm for 3 min (RT). The rates of reduction of the samples were used to calculate the amount of GSH and GSSG by interpolation of linear standard curves. In addition, the basal level of lipid peroxidation was measured in WT and null heart homogenates as total carbonyl content using OxyBlot kit (Chemicon, Inc).

To analyze protein-acrolein and protein-4-hydroxy-*trans*-2-nonenal (HNE) adducts, Western blots were probed with in-house raised rabbit polyclonal anti-protein-acrolein or anti-protein-HNE antibodies and thiobarbituric acid reactive species (TBARS) according to previously published methods.[5-7] In brief, hearts were homogenized in lysis buffer (25 mM Tris; 0.5 mM EDTA; 0.5 mM EGTA; protease inhibitor 1:100 dilution; phosphatase inhibitor, 1:100; pH 7.5), centrifuged (14,000x*g*, 15 min, 4°C), pellets re-suspended in lysis buffer supplemented with 1% NP-40, incubated for 4h[8], re-centrifuged, and supernatant used as membrane fraction. Total protein was measured using a commercially available kit (Bradford, Bio-Rad, Hercules, CA, USA). For Western blot analysis of protein-acrolein adducts, protein samples in 5x sample buffer (312.5 mM Tris base, pH 6.8 [Bio-Rad], 10% Glycerol, 11.5% SDS, 0.1% Bromphenol) supplemented with 50 mM NEM were separated under non-reducing conditions, whereas all

other proteins were separated under reducing conditions (buffer supplemented with 50 mM DTT). Briefly, heat-denatured (5 min, 95°C) protein samples were separated by SDS-PAGE and transferred to PVDF membranes (Bio-Rad). Membranes were processed by standard immunodetection techniques using a rabbit polyclonal antibody against KLH (Keyhole Limpet hemocyanin)-protein-acrolein adduct.[9] Polyclonal anti-KLH-acrolein antiserum was prepared as described by Uchida et al.[10] Briefly, KLH (1.0 mg) was incubated with acrolein (10 mM; freshly prepared from the acid hydrolysis of acrolein diethyl acetal) in 1.0 ml of 0.05M potassium phosphate, pH 7.2, for 24h at 37 °C. Excess acrolein was removed by gel filtration on a PD-10 column. The acrolein-modified KLH was mixed with Freud's complete adjuvant (1:1) and injected intradermally in New Zealand White rabbits. Boosters were repeated after 4, 8 and 12 weeks in Freund's incomplete medium. Immunoreactivity was detected by Western blotting using acrolein-modified BSA as an antigen. Actin or amido black staining was used as loading control. Quantification of band intensities was performed using Image Quant TL software (Amersham Biosciences).

In situ Model of Infarction: The left anterior descending coronary artery was ligated for 15 min or 30 min and reperfused for 15 min, 4h or 24h depending on the endpoints being measured.[11, 12] After reperfusion, mice were euthanized with sodium pentobarbital (0.1 ml; 40 mg/ml, i.p.), blood collected *via* cardiac puncture, and hearts perfused with TTC and Evans Blue for measuring infarction area, snap frozen (15 min or 4h reperfusion), or formalin-fixed (24h reperfusion) for biochemistry or immunostaining, respectively.

Langendorff Mode: Hearts were isolated and perfused in Langendorff mode (constant pressure ~80 mm Hg) with Krebs-Henseleit buffer (Sigma KH buffer: NaCl 118, KCl 4.7, KH_2PO_4 1.25, $MgCl_2$ 1.25, $CaCl_2$ 2.5, EDTA 0.5, $NaHCO_3$ 25, glucose 10; pH 7.4; 37 ºC) for 30 min prior to 15 min or 30 min ischemia without or with reperfusion for 15, 30 or 45 min as indicated. During perfusion, total flow and left ventricular pressure were recorded using an inline flow probe and meter (ME1PXN, T402/TS410; Transonic Systems Inc., Ithaca, NY) and a custom-made, plastic wrap balloon, respectively. The balloon was inserted into the left ventricle via the mitral valve and connected to a pressure transducer (APT300, Harvard Apparatus; Holliston, MA), a MacLab 4/e A/D converter and recorded (100 Hz) on a PC (Chart v. 4.2.3;

ADInstruments, Colorado Springs, CO). Systolic, diastolic, ±dP/dt and heart rate were derived from LV pressure waveform. Perfusate was collected prior to ischemia and during reperfusion at 1, 2, 3, 4, 5, 10, 15, 20, 30, and 45 min for measurement of CK and LDH activities (Thermo-Electron).

Electron Paramagnetic Resonance Spin Trapping Measurement of Oxygen Radicals: After intraperitoneal induction of anesthesia (sodium pentobarbital, Abbott Laboratories, Abbott Park, Ill), the heart was excised, and the ascending aorta was cannulated and perfused at a constant flow (2 mL/min) with Krebs-Henseleit buffer. After 15 min ischemia, the spin trap, 5,5-dimethyl-1-pyrroline-N-oxide (DMPO; 1 M; Dojindo Laboratories, Kumamoto, Japan) in buffer containing 100 μM diethylenetriaminepentaacetic acid (DPTA; Sigma Aldrich) was delivered immediately upon reperfusion via infusion (100 μL/min) through a side arm located close to the heart. During reperfusion, 20s collections of the effluent were made until 5 min of reperfusion as indicated. On sample collection, each tube was immediately frozen in lN_2. Electron paramagnetic resonance spectra were recorded as described previously.[12]

Cardiac Myocyte Experiments.

Isolation, Cell Death and Protein-Acrolein Adducts: For myocyte isolation, hearts were perfused for 10 min followed by digestion with collagenase or Liberase Blendzyme I (Roche) to release myocytes. Isolated myocytes were introduced into Tyrode's bicarbonate buffer with increasing [Ca^{++}]. Viable cells were incubated in laminin-coated plates overnight. Cardiomyocytes were superfused in an oxygenated-HEPES buffer without or with acrolein or with H_2O_2 (1, 5 or 10 μM) for 60 min. Similarly, myocytes were incubated with buffer only or acrolein (25 μM) for 15 min, after which acrolein was removed from all dishes, and the time-dependent removal of protein-acrolein adducts at 15, 30, 60 and 120 min post-acrolein was assessed in myocyte lysates by Western blotting as described above.

Using WT cells, preincubation with SN-6 (10 μM, 30 min; selective cardiac Na^+/Ca^{++} exchange [NCX1] inhibitor; Tocris Bioscience, Bristol, UK) was performed without and with subsequent acrolein (25 μM) exposure in HBSS. Digital images of myocytes (60-80/field) were taken at 5 min intervals for a 60 min exposure to quantify numbers of hypercontracted and total cells. Survival data were modeled using the Weibull survival distribution.[13] After λ and γ

parameters were described, these were used to find the mean lifetime using the formula: $\mu = \Gamma(1+1/\gamma)/\lambda$. Cardiomyocyte viability also was measured by post-exposure incubation with MTT reagent (500 μg/ml) and measurement of solubilized MTT absorbance at 595 nm as described previously.[14]

Electrophysiological Measurements: Sodium currents were recorded using the whole-cell patch clamp technique using an Axopatch-200B patch-clamp amplifier (Axon Instruments, Foster City, CA) at room temperature (21-22°C). Freshly isolated cardiomyocytes were pipetted into the perfusion chamber mounted on the stage of an inverted microscope. Whole cell access was attained using fire-polished borosilicate patch pipettes pulled on a Flaming-Brown horizontal puller (P-87, Sutter Instruments, Novato, CA), which ranged from 1.0 to 1.5 MΩ when filled with internal solution containing (in mM) CsCl 134, NaCl 10, $MgCl_2$ 1, Na_2-ATP 1, HEPES 10, EGTA 10, adjusted to pH 7.2 with CsOH. Cells were superfused at 1 to 1.5 mL/min with bath solution containing (in mmol/L) NaCl 20, CsCl 129, $CaCl_2$ 1.8, $MgCl_2$ 1, HEPES 10, $LaCl_3$ 0.5, adjusted to pH 7.4 using CsOH. Cs^{-2} and La^{+3} were used to block K^+ and Ca^{+2}-dependent currents, respectively.

Acrolein (50 μM; made fresh daily by acid hydrolysis of acrolein diethyl acetal; Sigma-Aldrich) dissolved in PBS or PBS vehicle alone was added to the bath solution immediately prior to the experiment. Upon attaining whole cell access by brief suction pulses, the cell was held at -95mV and cell capacitance and series resistance were estimated from a small depolarizing pulse (5mV) using Clampex software (Axon Instruments). Following breakthrough, series resistance for all analyzed cells was 4.8 ± 0.4 MΩ (SEM), and was compensated by 85%. To account for any slight changes in series resistance, compensation was readjusted preceding each recording. For analysis of the steady state activation (m_∞) of I_{Na}, I_{Na} was determined by depolarizing cardiomyocytes from a -95mV holding potential to various test pulses ranging from -80 to +20 mV, in 5mV steps lasting 50 ms. The sodium conductance (g_{Na}) of the maximal current ($I_{Na,}$ peak) at each potential (V) was calculated according to equation 1:

Eq. 1: $g_{Na} = (I_{Na,\,peak}) / (V-V_{rev})$

where V_{rev} was the reversal potential during each recording. The V_{rev} was calculated individually for each recording. Values of m_∞ were calculated by fitting the normalized g_{Na} values to a Boltzmann model according to equation 2:

Eq. 2: $m_\infty = [1+e^{(Vm-V)/s}]^{-1}$

where V_m is the membrane voltage at half-maximal m_∞ and s is the slope factor.

The voltage dependence of steady-state inactivation of I_{Na} was calculated with data generated by using the classic two-pulse protocol.[15] Cardiac myocytes were depolarized to -25mV for 50 ms from a holding potential of -95 mV (test pulse). The test pulse was preceded by a 500 ms conditioning pulse, which varied from -120 to -45 mV, in 5 mV increments. The ratio of the current elicited with and without the test pulse was found at each conditioning pulse potential, and was also fitted by the Boltzmann model, equation 3:

Eq. 3: $h_\infty = [1+e^{(Vh-V)/k}]^{-1}$

where V_h is the membrane voltage at half-maximal h_∞ and k is the slope factor.

The effect of acrolein (50 μM) on $V_{1/2\text{-act}}$ was determined by comparing control values measured immediately prior to addition of acrolein with that determined after 20 min acrolein exposure. To confirm that any change obtained was not the result of rundown, time-control experiments were performed in which $V_{1/2\text{-act}}$ was measured before and after 20 min of vehicle (PBS) perfusion.

Cardiac Biochemistry. Hearts were weighed, and then snap frozen in lN_2 for Western blotting and biochemical analyses. Glutathione (reduced GSH) and thiobarbituric acid (TBARS) levels were determined spectrophotometrically using MDA as standard as in previously published methods.[5]

Cardiac acrolein measurement by ultra performance liquid chromatography -- mass spectrometry (UPLC-MS):

Sample preparation. Hearts were excised from mice and Langendorff perfused for 30 min without or with 15 min ischemia. Hearts were snap frozen in lN_2 and stored at -80 °C until

processed. Heart tissue was pulverized with a stainless steel Bessman tissue pulverizer (Spectrum Labs, Rancho Dominguez, CA) after chilling with lN_2. Approximately 50 mg powdered tissue was placed in the bottom of a chilled glass Kontes Duall tissue grinder (Kimble-Chase, Vineland NJ) and a 20 % v/v extraction/reaction solution was added adjusted for estimated tissue and perfusion water (40 µL). The extraction/reaction solution was N-[2-(aminooxy)ethyl]-N,N-dimethyl-1-dodecylammonium iodide **(**QDA; 1 mM), $^{13}CD_3$ QDA-acrolein (8 nM), anthranilic acid (20 mM) in 50 % ammonium formate (100 mM, pH 5) and 50 % acetonitrile. After homogenization, the solution was transferred to a glass culture tube, capped, and allowed to react for 120 min at room temperature. At the end of the incubation, the solution was transferred to a plastic centrifuge tube and centrifuged for 10 min at 16,000x*g* to clarify the solution. The supernatant was transferred to a Waters maximum recovery vial, capped and placed into the Acquity autosampler chamber kept at 10 °C.

UPLC Protocol. From each sample, 10 µL was injected onto an Acquity UPLC HSS T3 column and concentration was interpolated from the calibration curve run in the same batch. The compounds were eluted from the column kept at 40 °C and into the mass spectrometer using a binary solvent system consisting of ammonium formate (50 mM) for A and formic acid (30 mM) in acetonitrile for B. The gradient profile was adapted after Breitbach et al., (2014): Initial conditions were 95:5 A:B ramping to 40:60 A:B over 1 min then ramping to 5:95 A:B at 3 min. From 3 to 4 min, B was ramped to 1:99 A:B and held until 4.5 min. The solvents were ramped back to the initial 95:5 A:B over 1 min and the column was re-equilibrated to 8 min. The flow rate of solvents was 0.55 mL/min.[16]

Acrolein derivatization and MS. N-[2-(aminooxy)ethyl]-N,N-dimethyl-1-dodecylammonium iodide (QDA) and *QDA ($^{13}CD_3$-labeled QDA, internal standard) were used to covalently bond with acrolein.[17] Free acrolein was made by acid hydrolysis of diethyl acetal acrolein (Sigma) in HCl (0.1 M) with a reaction time of 30 min at room temperature. The reaction conditions for conjugation were as follows: QDA (1 mM) or $^{13}CD_3$QDA (1 mM) was added to anthranilic acid (20 mM) in a solution of 50 % ammonium formate (100 mM, pH 5) in water and 50% acetonitrile; then free acrolein was added to a final concentration of 100 nM. The reaction was kept at room temperature for 120 min. The reaction products were then infused into a Waters (Milford, MA) Quattro Premier XE mass spectrometer under optimized ionization conditions and optimal daughter ions were used to program sensitive multiple reaction monitoring (MRMs). The

MRM transitions were: parent QDA-acrolein: 311.2→58.5, 311.2→97.95; parent *QDA-acrolein: 315.2→62.5, 315.2→98.2 (see Fig. 5Ai,ii).

Standard curve and sensitivity. Calibration solutions were made with concentrations starting at 100 pM to 90 nM QDA-acrolein (plus a fixed 8 nM concentration of *QDA-acrolein) in 50 % ammonium formate (100 mM, pH 5) and 50 % acetonitrile. These solutions were injected using a Waters Acquity UPLC. Each concentration was injected six times and the standard deviation for each was plotted and an equation was fitted to the data. The y-intercept was used to calculate the LOD of 0.4 fmol and the LOQ to be around 1 fmol. These data were used to construct a calibration curve: x-axis: concentration of QDA-acrolein; y-axis: the ratio of QDA-acrolein/$^{13}CD_3$ QDA-acrolein.

Cardiac Aldehyde Metabolism: GSTP-mediated metabolism of acrolein or 4HNE was measured in cardiac lysates (2 mg/ml) incubated with reduced glutathione (GSH, 100 μM) and acrolein or 4HNE (10, 20 or 100 μM). The amount of GS-conjugate was HPLC separated and measured on ESI-MS by comparison with a known quantity of added GS-^{13}C-propanal or GS-^{13}C-HNE (15 nmoles). To measure concentration and time dependence of GS-acrolein formation catalyzed by cardiac GSTP, cardiac homogenates were prepared in 0.1M phosphate buffer (pH 6.5). Lysate was passed through a PD-10 column using 0.1M phosphate buffer (pH 6.5). Protein level was measured by Lowry's assay (or Bradford's) and glass tubes were prepared with either 0 or 2 mg/ml protein in 0.1M phosphate buffer, pH 6.5 (total volume < 500 μl). To begin time or concentration assay, GSH was added (f.c. 100 μM) and then acrolein (f.c. 10-100 μM). At desired time, an equal volume of 20% TCA was added to stop reaction. Samples were centrifuged (13,000 rpm, 5 min, 4 °C). To the supernatant, ^{13}C-acrolein-GS (oxopropyl glutathione; 15 nmol; Sigma-Aldrich) was added. Total volume was increased to 1 ml with water and injected into HPLC (Waters). GS-conjugate was collected (retention time, approximately 20-22 min), fractions were dried (Speedvac), and then reconstituted in 1:1 H_2O:acetonitrile (100 μl, 0.1% acetic acid). Aliquots were injected on ESI/MS in positive ion mode and ion ratios of *m/z* 364 [^{12}C] and 367 [^{13}C] for acrolein and similarly for ^{12}C:^{13}C-GS-HNE conjugates, *m/z* 544 [^{12}C] and 547 [^{13}C] of WT and GSTP-null were compared.

JNK/c-Jun Assay: To assess the direct effects of *mGstp1/p2* gene deletion in the heart, the effects of I/R on MAPK signaling were assessed. Mice were anesthetized with sodium pentobarbital and heparin (80 U/kg, 0.2 ml, i.p.) before *in situ* I/R as above. After 15 min, hearts

were snap-frozen in lN_2 for subsequent analysis of MAPK activation and protein-acrolein adducts by Western blot as described above. Because of the known relationship between GSTP and JNK, the effects of I/R (15 min ischemia, 15 min reperfusion) on activation (phosphorylation) of JNK and c-Jun (JNK activity assay) were measured in homogenates using commercial antibodies (Cell Signaling Technology).[3]

Histology and Immunohistochemistry. General histology was performed on 10% formalin-fixed, paraffin-embedded tissue sections (4 μm) stained with H&E, rabbit polyclonal primary antibody against human GSTP1 (1:1,500; Novocastra)[18], IgG-purified polyclonal rabbit anti-protein-acrolein antibody (1:1,000; IgG-purified preimmune rabbit serum served as negative control)[3] or murine anti-myeloperoxidase antibody (MPO, Ab-1; Thermo Fisher Scientific)[3]. Secondary antibody was an anti-rabbit goat antibody used with a Vector Elite staining kit (HRP-tagged) using diaminobenzadine (DAB) as chromagen (1:1000; Vector). Images of mid-heart cross sections were made using a digital Spot camera mounted on an Olympus microscope and analyzed using Metamorph (Molecular Devices) or Image J (NIH).

Calculations and Statistics: Western blots were detected using a Typhoon 9600 and analysed band density of interest was normalized to actin or amido black band density. Values shown are means ± SE. Additional group data were compared using paired or unpaired *t*-test or One Way ANOVA with repeated measures and Bonferroni post-test where appropriate (SigmaStat, SPSS, Inc., Chicago, Il). Significance level in all treatments was set at $P<0.05$.

Chemicals and Solutions:

All chemicals were purchased from Sigma Chemical Co. (St. Louis, MO) unless otherwise noted.

Online Materials, Legends of Figures:

Online Figure I: Antioxidant proteins and basal protein-HNE adducts in GSTP-null hearts. **A**, Western blotting of antioxidant proteins (aldose reductase, AR; gamma cysteine ligase, GCL; thioredoxin, TRX); **B**, inducible cardioprotective proteins (iNOS; heme oxygenase 1, HO-1); and, **C**, protein-HNE adducts in cardiac lysates of naïve male WT and GSTP-null hearts. +CON, positive control.

Online Figure II: Ischemia-reperfusion injury in Langendorff-perfused GSTP-null hearts. **A,** Representative left ventricular (LV) pressure traces from (**i**) WT and (**ii**) GSTP-null hearts following 30 min perfusion (P), 30 min ischemia (I) and 45 min of reperfusion (R). **B,** Summary data of level of LV ischemic hypercontracture after 30 min of ischemia prior to onset of reperfusion (n=6 hearts per group).

Online Figure III: Cardiac myocyte toxicity to acrolein and H_2O_2. Summary of MTT absorbance in isolated cardiac myocytes of WT and GSTP-null hearts superfused with buffer (control, 0), **A,** acrolein (1-10 μM) or **B,** H_2O_2 (1-10 μM) for 60 min, and then incubated with MTT reagent for an additional 60 min. * $P<0.05$ significant difference between control and 10 μM acrolein or H_2O_2 in WT and GSTP-null, respectively (n=3 isolations per group).

Online Figure IV: Protein-acrolein adduct removal in WT and GSTP-null cardiac myocytes. A, Western blot of protein-acrolein adducts induced by acrolein (25 μM; 15 min) and assessed at 0, 15, 30, 60 and 120 min post-removal. **B, i,** Basal level (pre) of protein-acrolein adduct (250 kDa M_r band) in WT and GSTP-null cardiac myocytes. **B, ii,** Time-dependent change in protein-acrolein adducts (250 kDa M_r band). The intensity of the protein-acrolein adduct band at each time is normalized to the intensity of the band 15 min after acrolein formation indicated as post-acrolein time 0. * $P<0.05$ significant difference by Dunnett's repeated measure between 0 and 60 and 120 min post-acrolein in WT myocytes (n=3 isolations per group).

Online Figure V: Schematic of GSTP-mediated cardioprotection in ischemia/reperfusion (I/R). I/R increases ROS production leading to an increase in lipid peroxidation (LPO) and the formation of aldehydes (RCHO) such as acrolein. GSTP detoxifies acrolein by catalyzing the conjugation of acrolein with glutathione. This conjugate (GS-RCHO) is extruded from the heart. Acrolein toxicity likely involves increased protein crosslinking (protein-RCHO) and altered Na^+ channel (green) function promoting calcium overload via the Na^+-Ca^{2+} exchanger (pink) followed by cell hypercontracture and death.

Online Table I: Primers for quantitative real-time PCR of cardiac GSTs in male WT and GSTP-null mice.

Primer	Sequence
f-mGSTP1-qRTPCR	5’ ATGCCACCATACACCATTGTC 3’
r-mGSTP1-qRTPCR	5’ GGGAGCTGCCCATACAGAC 3’
f-mGSTalpha4-qRTPCR	5’ TGATTGCCGTGGCTCCATTTA 3’
r-mGSTalpha4-qRTPCR	5’ CAACGAGAAAAGCCTCTCCGT 3’
f-mGSTmu4.1-qRTPCR	5’ AGCTCACGCTATTCGGCTG 3’
r-mGSTmu4.1-qRTPCR	5’ GCTCCAAGTATTCCACCTTCAGT 3’
f-mGAPDH-qRTPCR	5’ AACTTTGGCATTGTGGAAGG 3’
r-mGAPDH-qRTPCR	5’ GGATGCAGGGATGTTCT 3’

Online Table II: Antioxidant defenses in the heart of GSTP-null mice.

Antioxidant	**WT (n=4-7)**	**GSTP-null (n=4-7)**
Superoxide dismutase (U/mg protein)	12.63 ± 0.38	11.51 ± 0.23*
Catalase (μmoles/min/mg protein)	15.5 ± 0.7	14.5 ± 0.37
Glutathione Reductase (nmoles/min/mg protein)	7.74 ± 0.45	7.94 ± 0.26
Glutathione Peroxidase (H_2O_2) (nmoles/min/mg protein)	19.34 ± 1.54	19.41 ± 1.41
Glutathione Peroxidase (cumene hydroperoxide) (nmoles/min/mg protein)	9.95 ± 0.96	10.32 ± 0.51
Glutathione (reduced; μmol/g wet wt)	1.7±0.3	1.9±0.3
Glutathione (reduced; μmol/g protein)	42.6±5.6	50.8±6.6

Values are means ± S.E. * P< 0.05 vs. matched WT.

Online Table III: Cardiac parameters of male wild-type (WT) and GSTP-null mice.

Parameter	**WT (n=15)**	**GSTP-null (n=16)**
Body Weight (g)	26.5±0.6	24.6±0.4
Heart/BWT (mg/g)	5.33±0.30	5.22±0.14
Left Ventricle/BWT (mg/g)	3.80±0.22	3.70±0.14
Heart Rate (bpm)	495±15	498±13
LV End Diastolic Volume (μl)	27.5±1.9	33.6±1.8*
LV End Systolic Volume (μl)	7.6±0.5	8.4±0.8
Ejection Fraction	0.72±0.01	0.75±0.01*
LV Systolic Pressure (mm Hg)[a]	91±4	89±3
LV Diastolic Pressure (mm Hg)[a]	5.9±0.9	7.8±1.5

Values are means ± S.E. [a] LV systolic pressure and LV diastolic pressure (n=6-7);

* $P<0.05$ vs. age-matched WT.

Online Table IV: Ischemia-induced aldehyde content in Langendorff-perfused GSTP-null hearts.

Aldehyde	WT (P; n=4)	WT (I; n=4)	Null (P; n=4)	Null (I; n=4)
Malondialdehyde (MDA)	3.51±1.43	3.99±0.26	3.33±1.18	4.66±0.79
Hexanal (HEX)	0.178±0.090	0.177±0.092	0.123±0.063	0.166±0.118
Hydroxynonenal (HNE)	0.086±0.036	0.071±0.017	0.063±0.045	0.062±0.019

P, perfusion only; I, perfusion for 30 min prior to 15 min global ischemia. Values are means ± S.D. in pmol/mg heart wet wt. n = number of mice.

Online Table V: Ischemia-induced changes in glutathione levels in Langendorff-perfused GSTP-null hearts.

Aldehyde	WT (P; n=3)	WT (I; n=3)	Null (P; n=3)	Null (I; n=3)
Reduced Glutathione (GSH)	1.36±0.09	1.23±0.01§	1.01±0.07*	1.80±0.17
Oxidized Glutathione (GSSG)	0.018±0.004	0.030±0.004	0.030±0.002*	0.050±0.005
GSH/GSSG Ratio	79±19*§	40±5	31±1	35±7

Perfusion only, P, 45 min; Ischemia, I, perfusion for 30 min prior to 15 min global ischemia. Values are means ± S.E. in nmol/mg heart wet weight. * $P<0.05$ between P and I; § $P<0.05$ between WT and matched GSTP-null by One Way ANOVA. n = number of hearts.

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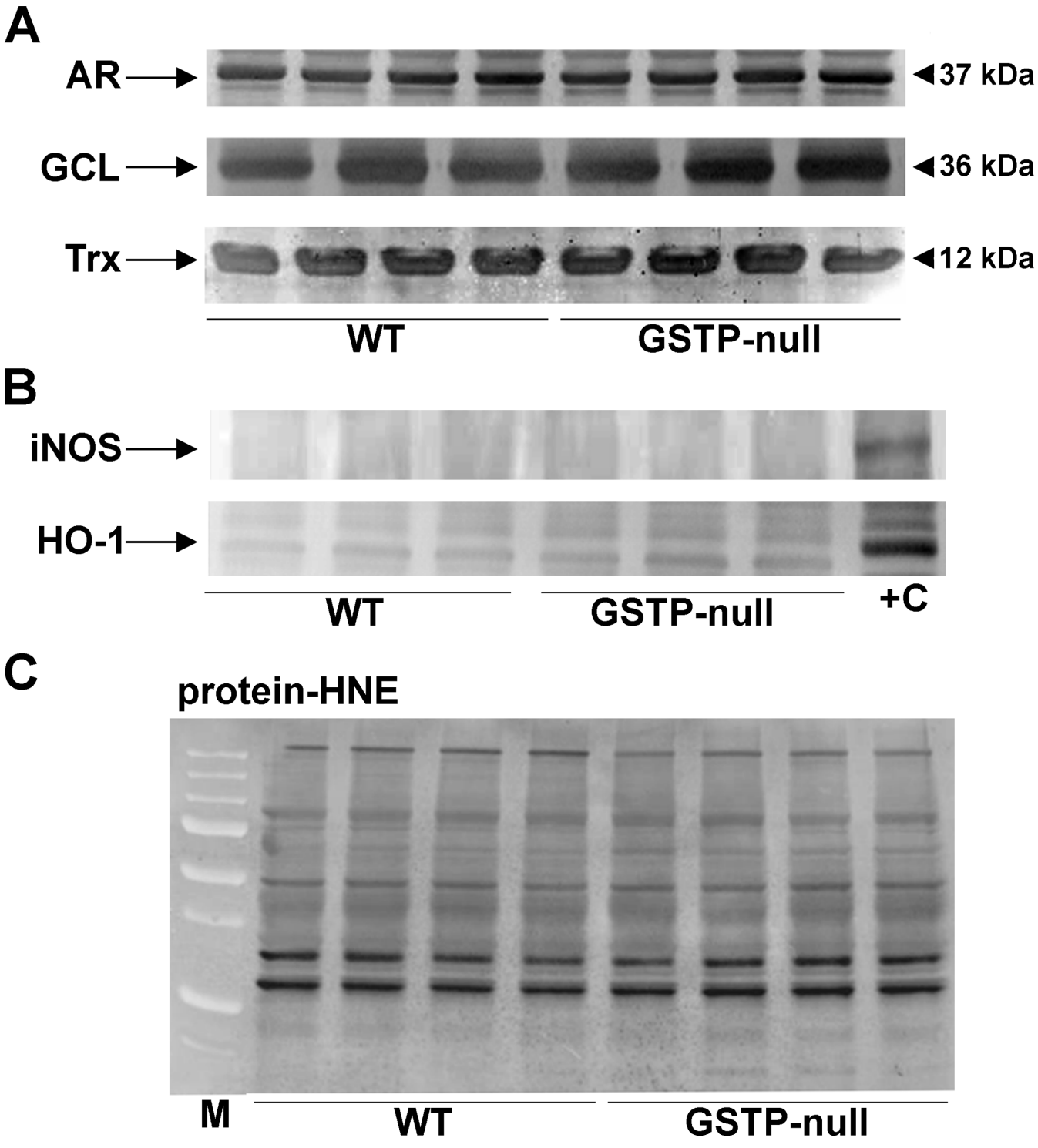
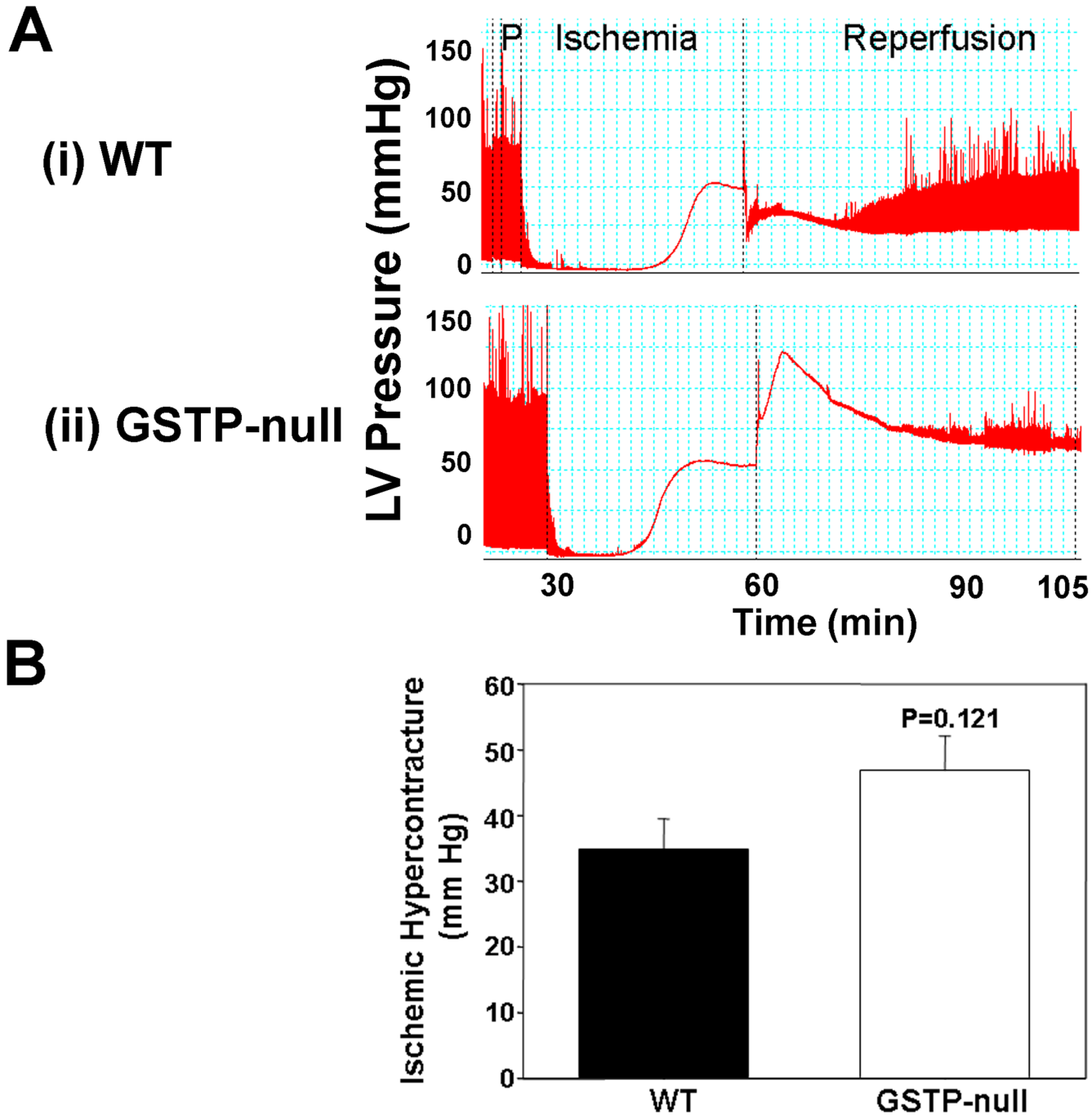
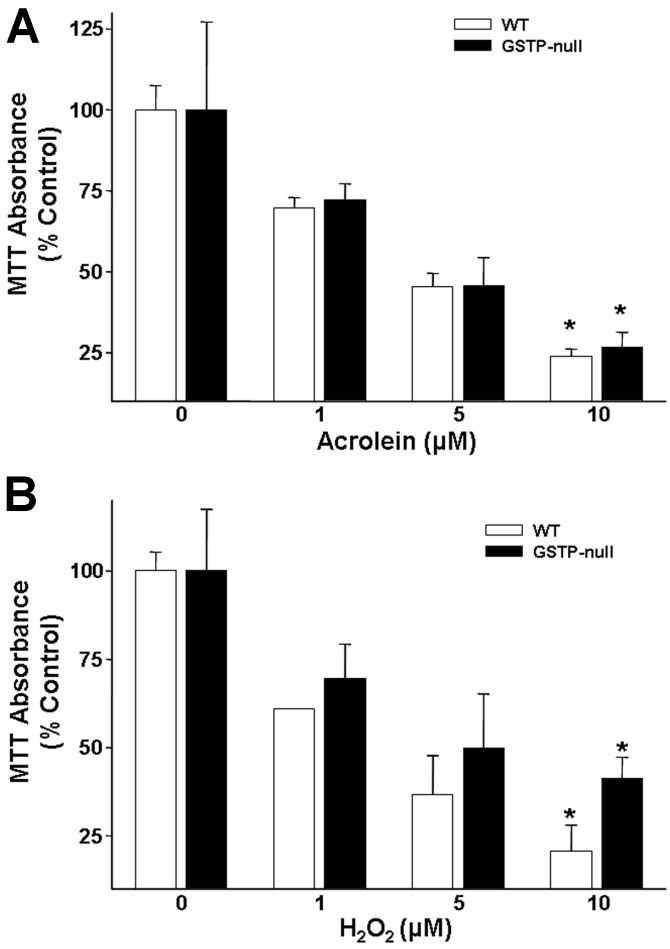
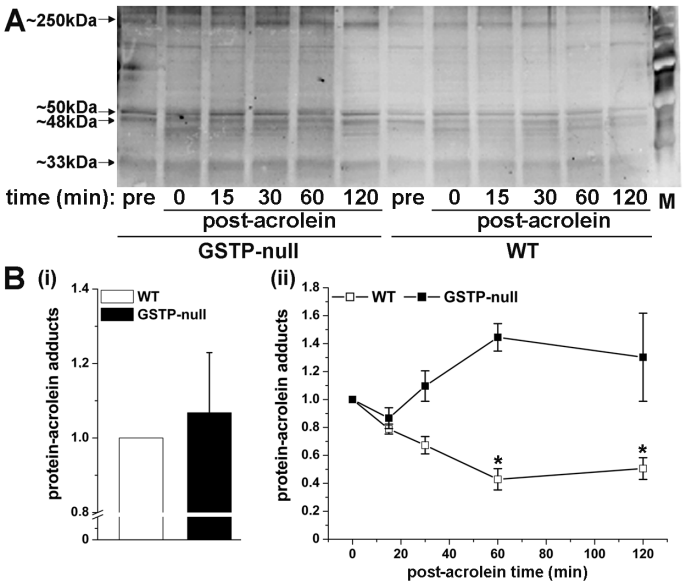
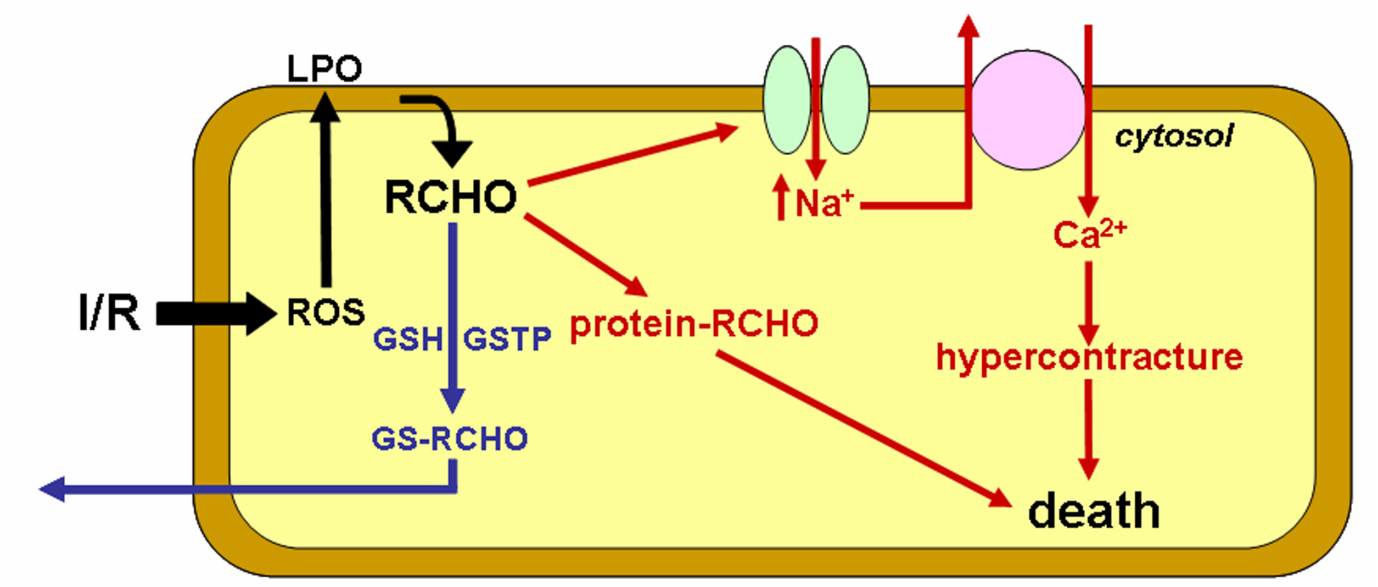

A
AR
37 kDa
GCL
36 kDa
Trx
12 kDa
WT
GSTP-null
B
iNOS
HO-1
WT
GSTP-null
+C
C
protein-HNE
M
WT
GSTP-null


A
(i) WT
(ii) GSTP-null
P
Ischemia
Reperfusion
LV Pressure (mmHg)
150
100
50
0
150
100
50
0
30
60
90
105
Time (min)
B
Ischemic Hypercontracture (mm Hg)
60
50
40
30
20
10
0
P=0.121
WT
GSTP-null


A
WT
GSTP-null
MTT Absorbance
(% Control)
125
100
75
50
25
0
1
5
10
Acrolein (μM)
B
WT
GSTP-null
MTT Absorbance
(% Control)
100
75
50
25
0
1
5
10
H2O2 (μM)


A
~250kDa
~50kDa
~48kDa
~33kDa
time (min): pre 0 15 30 60 120 pre 0 15 30 60 120 M
post-acrolein
post-acrolein
GSTP-null
WT
B (i)
WT
GSTP-null
protein-acrolein adducts
1.4
1.2
1.0
0.8
0
(ii)
WT
GSTP-null
protein-acrolein adducts
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
0
20
40
60
80
100
120
post-acrolein time (min)
*
*


LPO
RCHO
I/R
ROS
GSH
GSTP
GS-RCHO
protein-RCHO
Na+
cytosol
Ca2+
hypercontracture
death