INFLUENCE OF GLUTATHIONE-S-TRANSFERASE (GST) INHIBITION ON LUNG EPITHELIAL CELL INJURY: ROLE OF OXIDATIVE STRESS AND METABOLISM

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ABSTRACT

Oxidant-mediated tissue injury is key to the pathogenesis of acute lung injury (ALI). Glutathione-S-Transferases (GSTs) are important detoxifying enzymes that catalyse the conjugation of glutathione with toxic oxidant compounds and are associated with acute and chronic inflammatory lung diseases. We hypothesised that attenuation of cellular GST enzymes would augment intracellular oxidative and metabolic stress and induce lung cell injury. Treatment of murine lung epithelial cells with GST inhibitors, ethacrynic acid (EA) and caffeic acid compromised lung epithelial cell viability in a concentration-dependent manner. These inhibitors also potentiated cell injury induced by hydrogen peroxide (H_2O_2), tert-butyl-hydroperoxide and hypoxia-reoxygenation (HR). SiRNA-mediated attenuation of GSTπ but not GSTμ expression reduced cell viability and significantly enhanced stress (H_2O_2/HR)-induced injury. GST inhibitors also induced intracellular oxidative stress (measured by dihydrorhodamine 123 (DHR123) and dichlorofluorescein (DCF) fluorescence), caused alterations in overall intracellular redox status (as evidenced by NAD^+/NADH ratios) and increased protein carbonyl formation. Furthermore, the antioxidant N-acetylcysteine completely prevented EA-induced oxidative stress and cytotoxicity. While EA had no effect on mitochondrial energetics, it significantly altered cellular metabolic profile. To explore the physiological impact of these cellular events, we used an *ex vivo* mouse isolated perfused lung model. Supplementation of perfusate with EA markedly affected lung mechanics and significantly increased lung permeability. The results of our combined genetic, pharmacological and metabolic studies on multiple platforms suggest the importance of GST enzymes, specifically GSTπ, in the cellular and whole lung response to acute oxidative and metabolic stress. These may have important clinical implications.

INTRODUCTION

Acute lung injury (ALI) and acute respiratory distress syndrome (ARDS) are life-threatening inflammatory disorders which may result from both medical and surgical situations such as sepsis, heart surgery and lung transplantation (41). Among these, ischemia and reperfusion injury constitutes a unique entity, wherein metabolic and oxygen starvation and subsequent re-oxygenation can lead to the release and accumulation of reactive oxygen species (ROS) (4). ROS can directly oxidise and damage DNA, protein and lipid membranes, inactivate antioxidant enzymes and enhance expression of pro-inflammatory genes, ultimately leading to cell injury and apoptosis (11).

The Glutathione-*S*-Transferase (GST) antioxidant enzymes form a multifunctional superfamily of intracellular isozymes that catalyse the conjugation of reduced glutathione (GSH) with a variety of toxic compounds including xenobiotics and oxidative intermediates (such as lipid and DNA hydroperoxides and aldehydes) (17, 18). This renders them less toxic and facilitates their removal from the cell (17, 18). Their key role in cellular detoxification protects macromolecules from attack by reactive electrophiles, such as ROS products, environmental carcinogens and chemotherapeutic agents. Increasing evidence suggest that GST expression and function are important in both cancer progression (24, 39) and acute inflammatory conditions (23). For example, Luo et al. demonstrated that treatment of mice with recombinant GSTP1 attenuated LPS-induced acute inflammation (23).

The clinical importance of diminished endogenous GST expression and activity is emerging. At least five of the eight GST genes in humans have functional polymorphisms where substitutions or deletions affect enzyme expression and activity

and are present in up to 50% of the population (16). Such polymorphisms are believed to contribute to individual differences in the clearance of xenobiotics and oxidative stress products, and to susceptibility to various inflammatory and proliferative pathologies (16). For instance, individuals with homozygous GSTM1 gene deletions appear to be more susceptible to DNA damage induced by GSTM1 substrates (32) and are more at risk of developing lung adenocarcinoma (7). Preliminary clinical studies also suggest that attenuated GST expression may contribute to ALI (25). Moradi and colleagues showed that GSTM1 polymorphisms were associated with increased mortality in critically ill patients with ALI/ARDS (25). In the setting of lung transplantation, donor GST polymorphisms dramatically increased the development of primary graft dysfunction (PGD) (8, 14). Despite these suggestions, the exact role of GST attenuation in lung cell injury, the basic mechanisms involved and the relative impact of different GST isozymes are not known.

We hypothesised that GST attenuation would lead to increased intracellular oxidative stress affecting mitochondrial energetics and cellular metabolism resulting in lung cell injury. To test these ideas, we studied the influence of pharmacological inhibitors ethacrynic acid (EA) and caffeic acid (CA) (1, 28, 29, 37, 49) and RNA silencing of GST isoforms on lung epithelial cell survival, ROS generation and energetic/metabolic status in murine cell culture models of ALI. Finally, the physiological impact of GST inhibition was also explored using an *ex vivo* murine isolated perfused lung model by focusing on potential effects on lung mechanics and permeability.

MATERIALS AND METHODS

Cell culture

Murine lung epithelium MLE-12 cells (ATCC, Rockville, MD) were grown in Hanks medium (F12:MEM) supplemented with 1% penicillin/streptomycin and 5% fetal calf serum at 37°C in humid conditions with 5% CO_2, 95% air. For hypoxia and reoxygenation (HR) experiments, we used deoxygenated culture medium (bubbled with 95% N_2 and 5% CO_2 gas mixture under sterile conditions) and a hypoxic chamber (Mini Galaxy A, Wolf Laboratories, York, UK). Cells were exposed to hypoxic conditions for 12 or 24 h. Subsequently, media was exchanged for fresh, pre-warmed, oxygenated media and cells were exposed to normoxic conditions for increasing periods of reoxygenation (2, 6, 12, 24 h).

GST inhibition: method and efficacy

The pharmacological agents ethacrynic acid (EA) and caffeic acid (CA) were employed to attenuate activity of purified bovine liver GST (350 mUnits) or endogenous GSTs in cell lysates. The degree of inhibition was evaluated using the GST fluorometric activity assay kit (Biovision, Mountain View, CA, USA). A linear increase in fluorescence at excitation/emission (ex/em) 380/460 nm was measured over the initial 10-min period and calculated as fluorescence units/min (FU/min). A standard calibration curve was used to determine GST activity (mU) in unknown samples.

To complement the pharmacological data we employed RNAi to specifically knockdown GST μ or π isoforms. This was achieved using pre-designed GST μ and π ON-TARGETplus smartpool siRNA (Dharmacon siRNA Technologies, Thermo Scientific, UK). In brief, 20 pmol of scrambled control or GST-targeting siRNA was

introduced into MLE cells using lipofectamine 2000 (Invitrogen). After 5 h, the 'siRNA-lipofectamine' media was replaced with fresh media and cells were incubated for an additional 48 h, prior to further experimentation.

The extent of siRNA-mediated knockdown of GST expression was evaluated by RT-PCR. Total RNA was extracted from transfected cells using the RNAeasy mini kit (Qiagen, Valencia, CA, USA) (13) and quantified spectrophotometrically (Biophotometer plus, Eppendorf, UK). Single-stranded cDNA was synthesized from 2 µg RNA with TaqMan RT-PCR reagents using GeneAmp PCR System 9700 (Applied Biosystems, Warrington, UK) (13). The cycling conditions were 25°C for 10 mins, 48°C for 30 mins, and 95°C for 5 mins. cDNA was amplified using the TaqMan gene expression master mix (Applied Biosystems) and TaqMan probes specifically targeted against mouse GSTµ (Mm00833915_g1) and GSTπ (Mm00496606_m1) (Applied Biosystems). A standard nucleic acid template and water were used as positive and negative controls, respectively. Variations in RNA loading between reactions were normalised by measurement of 18S ribosomal RNA (12). PCR was performed under recommended thermal cycling conditions. Initially 50°C for 2 mins and 95°C for 10 mins (for enzyme activation), followed by 40 cycles of 95°C for 15 sec (cDNA denaturation) and 60°C for 1 min (probe annealing and extension). Data was collected using the ABI PRISM 7700 and Sequence Detection System 1.9.1 (Applied Biosystems) and analysed using the comparative cycle threshold (Ct) method (5).

Protein expression was evaluated by western blotting. In brief, protein extracts (50 µg) were separated on 10% SDS-polyacrylamide gels, transferred to nitrocellulose membranes and incubated overnight with polyclonal anti-GSTµ (1:500) or anti-GSTπ (1:500) antibodies (Abcam, UK). Antibody binding was detected using peroxidise-

conjugated anti-goat IgG (1:2000; Cell Signaling Technology, New England Biolabs, UK) and chemiluminescence detection reagents (Amersham Biosciences, UK). Equivalent loading of protein samples was confirmed by mouse monoclonal anti-β-actin or anti-α-tubulin IgG (1:2000; Sigma).

Analysis of lung cell viability

The effect of GST inhibition on cell viability was evaluated under control conditions and in combination with known oxidative stress conditions: hydrogen peroxide (H_2O_2), tert-butyl hydroperoxide (tBH) or HR. Cell viability was analysed by the MTT reduction assay (26). The optical density was measured as an index of cell viability, at 595 nm using an MRX II plate reader (Magellan Biosciences, UK).

To delineate the involvement of apoptosis versus necrosis we employed the Annexin V-FITC, Propidium Iodide (PI) apoptosis/necrosis detection kit (Sigma) and FACS calibur flow cytometer (Becton Dickinson) (38). Harvested cells were exposed to Annexin V-FITC and PI in binding buffer for 10 mins in the dark at room temperature. Ten thousand events were acquired and analysed using the CellQuest program. Cellular debris (with low forward and side light scatter) was excluded from the analysis. FITC and PI fluorescence were detected at 525 nm and 620 nm, respectively. Quadrant statistics display Annexin V and PI fluorescence on the x- and y- axis respectively, to identify viable cells (negative for both Annexin V and PI), apoptotic cells (positive for Annexin V, negative for PI) and late apoptotic dead cells (labelled with both Annexin V and PI). In most instances, apoptosis involves activation of caspase cascades. To further evaluate the role of apoptosis, cells were treated in the presence or absence of 100 μM zVAD (Sigma), a broad-spectrum caspase inhibitor and cell viability was assessed by MTT assay.

Detection of oxidative stress

The influence of GST inhibition on intracellular ROS was quantified using the ROS-sensitive fluorescent dyes dihydrorhodamine (DHR) and 2′,7′-dihydrodichloro-fluorescein diacetate (H_2DCF-DA) (Invitrogen). MLE cells were pre-incubated for 30 mins with 5 μM DHR or 20 μM H_2DCF-DA before exposure to the GST inhibitor EA for 1 h. Cells incubated in media alone or H_2O_2 served as negative and positive controls, respectively. Following treatment, cells were harvested and resuspended in FACS wash buffer (PBS supplemented with 5 mM EDTA, 2% FCS and 0.1% sodium azide). Samples were analysed by flow cytometry at ex/em 495/525 nm. ROS quantification is reported as Mean Fluorescent Intensity (MFI). To evaluate the role of specific ROS in EA-induced cytotoxicity, cells were pre-incubated for 1 h prior to EA treatment with the ROS scavengers N-acetyl-cysteine (NAC, 10 mM) or cell permeable PEG-conjugated catalase (5000 U/ml), or inhibitors of catalase (aminotriazole, ATZ, 10 mM), superoxide dismutase (diethyldithiocarbamate, DETC, 1 mM), NADPH oxidase (diphenyliodonium, DPI, 10 μM) and nitric oxide synthase (N-Nitro-L-Arginine Methyl Ester hydrochloride, L-NAME, 10 mM).

To evaluate the effect of GST inhibition on mitochondrial energetics (relative levels of ATP, ADP and AMP) and redox status (reflected by NAD and NADH ratios) (2, 21, 31), intracellular adenine nucleotides were extracted from control and experimental cells as described previously (47) and measured by reversed-phase HPLC and UV detection at 254 nm. Briefly, samples were extracted with ice-cold perchloric acid (0.4 M) and neutralized with equimolar KOH. $KClO_4$ was then precipitated by centrifugation and the supernatant was retained. Levels of ATP, ADP, AMP, NAD^+ and ADPR, a breakdown product of NADH following acid precipitation (21, 31) were analysed using

208 the Agilent HPLC 1100 system. The mobile phase was 0.1 M potassium dihydrogen
209 phosphate (pH 6.0) with 0.15 M potassium chloride as phase A and 15% acetonitrile in
210 A as phase B with the Hypersil BDS column and a flow rate 0.9 ml/min. The
211 concentration of each nucleotide was expressed as nmol/ml of extract with reference to
212 external standards used for calculation of concentrations with reference to cell protein.
213
214 Protein carbonyls were estimated using a 2,4-dinitrophenylhydrazine (DNPH)-based
215 procedure reported elsewhere (6, 33) with minor changes. Briefly, cell lysates
216 containing 0.1 mg protein were incubated with 0.1% DNPH for 1 h at room
217 temperature. Samples were precipitated by addition of 30% trichloroacetic acid.
218 Following centrifugation, pellets were washed with ethanol:ethyl acetate (1:1 v/v) and
219 dissolved in 8 M guanidine–hydrochloride, 13 mM EDTA and 133 mM Tris pH 7.4.
220 Optical density at 365 nm was determined using a Hitachi U-2000 UV–VIS
221 spectrophotometer. The carbonyl content was calculated using Beer's Law and an
222 extinction coefficient of $2.1\times10^4\ M^{-1}\ cm^{-1}$. Results are reported as nmol carbonyl/mg of
223 protein.
224
225 **Metabonomics**
226 Global metabolic changes associated with GST inhibition and oxidative stress, were
227 detected by metabonomic analysis (3). Cellular extracts from control and experimental
228 groups were prepared for NMR spectroscopy analysis. A Bruker DRX600 spectrometer
229 (Bruker Biospin, Rheinstetten, Germany) was employed, operating at 600.13-MHz ^1H
230 NMR frequency and 283 K. Raw spectra were phased, baseline corrected, calibrated to
231 the TMSP internal standard resonance (d_H = 0.00ppm) and imported into Matlab
232 (Student 2007, The MathsWorks, USA). Spectra were normalised to protein. Isolated
233 metabolite signals within spectra were defined through visual inspection and their peak

areas were determined. Metabolite assignment was made using the Madison Metabolomics Consortium Database (9).

Ex vivo 'Isolated Perfused Lung' model

To investigate the physiological relevance of our cellular findings, the direct impact of GST inhibitor EA on lung physiological parameters was explored using an isolated perfused mouse lung model. All procedures were approved by the United Kingdom Home Office in accordance with the Animals (Scientific Procedures) Act 1986, UK. Male C57BL6 mice (Charles River Laboratories, Margate, UK) aged 8 to 12 weeks were used for the ex-vivo isolated perfused lung (IPL) system (Hugo Sachs Elektronik – Harvard Appartus GmbH, March-Hugstetten, Germany). In brief, mice were anaesthetised, tracheostomised and ventilated with a custom-made mouse ventilator as previously described (35) at a respiratory rate of 80 breaths/min. After thoracotomy/laparotomy and heparinisation, animals were exsanguinated and the pulmonary artery and left atrium cannulated. Lungs were perfused in a non-recirculating manner with pre-warmed RPMI 1640 supplemented with 4% BSA and 1.4 g/L NaCl (Sigma) to increase osmolarity, as described previously (34). All experiments had an initial 15-min equilibration period, and low tidal volume (VT) of 8 ml/kg was applied throughout to minimise lung injury development. Peak inspiratory pressure (PIP), PEEP and gas flow were monitored continually via transducers, connected to a PowerLab data acquisition system (AD Instruments, Chalgrove, UK). Data was analysed using Chart software (version 5.5.6, AD Instruments). Accurate measurements of VT, respiratory system compliance (Crs) and resistance (Rrs) were determined by end-inflation occlusion at 30-min intervals.

Lung permeability index

The pulmonary endothelial/epithelial permeability to a fluorescence-labelled albumin was assessed, using an adaptation of a previously reported fluorescence-based method for measuring vascular leak into the alveolar space (45). Following a 2-h perfusion, 1 mg/ml Alexa Fluor 594-conjugated albumin (Invitrogen) was added to the perfusate in the recirculating circuit. After 30 mins, the perfusate was collected from the reservoir and stored in the dark. Thereafter the circuit was switched to a non-recirculating system for 5 mins to wash out the residual fluorescence from the lung vasculature. BALF was obtained as described previously (44). Fluorescence levels of perfusate, BALF and lung tissue were measured using a fluorescence plate reader (Flx-800, Bio-tek Instruments Inc., UK) at absorption 590 nm. An index of lung endothelial and epithelial permeability was then estimated by the ratio of lung tissue:perfusate and lavage fluid:perfusate fluorescent signal, respectively. Protein levels in BALF were also quantified as another index of pulmonary permeability.

Statistics

Data are reported as the mean of repeated experiments ± standard deviation (SD). The majority of statistical analyses were performed using Prism software (version 4.0, GraphPad, La Jolla, USA). Metabonomics data was analysed by SPSS 17 (Chicago, USA). Comparison of three or more variables was carried out by either one-way analysis of variance (ANOVA) or repeated measure ANOVA with multi-comparisons/post-hoc tests (including Bonferroni) where appropriate. Statistical significance of metabonomics data was assessed by Kruskal-Wallis test. Multiple comparisons of metabonomics data was controlled for using the Benjamini–Hochberg procedure, with accepted false discovery rate 0.05. A p value <0.05 was considered significant.

RESULTS

EA and CA inhibit GST activity and decrease lung cell viability

To study the efficiency of GST inhibition, pure GST and MLE cell lysates (prepared from $4x10^6$ cells) were incubated with increasing concentrations of GST inhibitors at 37°C for 1 h. Both EA and CA effectively inhibited GST activity in pure GST as well as MLE cell lysates in a concentration-dependent manner with an IC_{50} of approximately 25 μM and 1 mM, respectively. Exposure of MLE cells to increasing concentrations of EA and CA revealed major morphological changes and a concentration-dependent reduction in cell viability, assessed by MTT assay. After 5 h exposure, 100 and 300 μM EA reduced viability to 92 ± 7 and 13 ± 7 % of control (Fig. 1A). Similar findings were obtained following 24 h treatment with CA, which led to a 55 ± 35 % reduction in cell viability at 1 mM (Fig. 1B). To explore if GST inhibitor-induced cytotoxicity was limited to epithelial cells or applicable to other cell types in the lung, we have explored the effect of EA on primary mouse lung endothelial cells available in our laboratory. Exposure of these cells to increasing concentrations of EA for 5 hours showed that the endothelial cell viability was also compromised by 300 μM ethacrynic acid (74 ± 7 % of control).

Apoptotic nature of cell death

The nature of EA-induced cell death was investigated by staining MLE cells with Ann-V and PI and fluorescently labelled cells were identified using FACS (Fig. 2). Untreated cells showed 90 % cell viability, which decreased to 74 % and 17 % following 3 and 5 h incubation with 300 μM EA, respectively. This was associated with significant rise in the necrotic cell population, i.e. those exhibiting PI fluorescence in the absence of Ann-

V labelling. Moreover, after 5 h treatment the percentage of Ann V-fluorescent cells increased by >10-fold, indicative of an apoptotic cell population. Cells exhibiting both Ann V and PI increased from 1 to 57 % of total population.

To identify whether caspases are involved in EA-induced cell injury, MLE cells were incubated with zVAD (100 μM) prior to treatment with 0-300 μM EA (Fig. 3A). As a positive control, we evaluated if zVAD pre-incubation influenced cell death induced by a combination of tumour necrosis factor alpha (TNFα/cycloheximide treatment (Fig. 3B). Whilst zVAD pre-incubation attenuated cell death induced by TNF/cycloheximide (cell viability of 24 % vs. 5 %) it had no influence on EA-induced cell death.

Potentiation of stress-induced cytotoxicity by GST inhibitors

H_2O_2 and tBH alone resulted in a concentration-dependent reduction in cell viability over 5 h. These stress-induced cytotoxic effects were significantly potentiated by subtoxic concentrations of GST inhibitors namely 100 μM EA (Fig. 4A + 4C) and 0.5 mM CA (Fig. 4B). For instance, cell viability was reduced from 64 ± 24 % after 0.5 mM H_2O_2 treatment to 26 ± 14 % and 31 ± 5 % in the presence of EA and CA respectively, as compared to untreated controls. Similarly, EA exacerbated tBH-induced cell death. For instance following 1 mM tBH treatment, the presence of GST inhibitor reduced viability from 71 ± 4 % to 23 ± 14 % of untreated controls.

Prior to studying the influence of EA on MLE cell survival in the setting of hypoxia/reoxygenation (HR), we explored the influence of increasing duration of hypoxia and reoxygenation on cell viability. Interestingly, hypoxia up to 24 h and reoxygenation up to 6 h did not appear to compromise MLE cell viability measured as MTT activity (Fig. 4D). Injury however, was unmasked in the presence of EA. For

instance, 25 μM EA had no measurable effect during normoxia but it reduced viability of hypoxic control by 19 ± 9 %. In addition, 50 μM EA reduced cell viability by 35 ± 11 % in normoxic conditions, but led to a 77 ± 10 % decrease in viability during HR conditions.

Oxidative stress is involved in EA-induced cytotoxicity

To test the hypothesis that EA-induced cell injury is mediated by an altered intracellular redox state (1, 37), we initially monitored oxidative stress with redox-sensitive fluorescent dyes. Exposure of MLE cells to EA (300 μM) for 1 h increased the mean fluorescence intensity (MFI) of DHR123 and DCF by over 2- and 30-fold respectively, as compared with basal levels (Fig. 5A+B). In the control experiment, EA had no direct effect on dye autofluorescence in the absence of MLE cells after 3 h (data not shown).

To assess global intracellular redox state, nucleotides NAD^+ and ADPR (the breakdown product of NADH) were measured by HPLC and UV detection and their ratio calculated. Figure 5C shows that redox state was altered in a concentration dependent manner by EA exposure alone causing a decrease in NAD^+/NADH ratios to 11 % of untreated control by 300 μM EA. Furthermore, H_2O_2 also reduced NAD^+/NADH ratios and this effect was potentiated by EA (43 % vs. 28 % of untreated control following 3 h treatment with H_2O_2 in the absence or presence of 100 μM EA, respectively, $p<0.05$)(Fig. 5D).

To further evaluate intracellular oxidative stress in the setting of GST inhibition, excess formation of carbonylated protein was monitored spectrophotometrically. Exposure of cells to 100 μM EA alone did not affect steady state levels of protein carbonylation

(Fig. 5E). H_2O_2 alone increased protein carbonyls from 1.2 ± 0.2 to 2.6 ± 0.3 nmoles/mg and EA significantly enhanced H_2O_2-induced increase in protein carbonyls from 2 to 4-fold, as compared to untreated control after 3 h of treatment (Fig. 5E).

Inhibitors of H_2O_2 and NO do not attenuate EA-induced cell injury

We have attempted to further characterise the reactive species involved in EA-mediated lung cell injury. Specifically, to test the hypothesis that increased generation of H_2O_2 is responsible for EA-induced cell injury (37), we investigated the effect of modulating catalase activity by pre-incubating cells with either: cell-permeable (PEG-conjugated) catalase (5000 Units/ml); or the catalase inhibitor aminotriazole (ATZ, 20 mM). Although catalase completely abolished the cytotoxic effect of H_2O_2 (Fig. 6B), EA-induced cell injury was unaffected by PEG-catalase (Fig. 6A). ATZ-induced catalase inhibition also had no influence on EA-cytotoxicity (Fig 6C).

To further evaluate the role of intracellular oxidative stress in GST inhibitor-induced cytotoxicity, we pre-incubated cells with a variety of inhibitors affecting well know oxidative stress pathways and the broad-spectrum antioxidant NAC. Exposure of cells to L-NAME and DPI, targeting ROS generators nitric oxide synthase and NADPH oxidase respectively, had no impact on EA-induced cell death. However, cells pre-incubated with the ROS scavenger NAC (10 mM) fully protected cells from injury induced by 300 μM EA (92 ± 6 % vs. 13 ± 7 % of untreated control, $p<0.05$)(Fig. 7C) and 1 mM CA (84 ± 6 % vs. 45 ± 35 % of untreated control, $p<0.05$)(Fig. 7D). Furthermore, pre-treatment with NAC completely abolished EA-induced increased fluorescence (MFI) of ROS-sensitive dyes DHR123 and DCF (45 ± 8 vs. 14 ± 2 and 352 ± 49 vs. 9 ± 1, $p<0.05$) (Fig. 7 A and B).

388 **GST inhibition has no influence on mitochondrial energetics**

389 To investigate whether EA mediates cellular injury through oxidative stress affecting
390 mitochondrial energetics, we determined ATP/ADP nucleotide ratios in extracts of cells
391 exposed to EA, H_2O_2 or both treatments over 3 h and 5 h. In control cells ATP levels
392 were 27.6 ± 1.7 nmol/mg protein and ADP levels were 2.1 ± 0.3 nmol/mg protein.
393 ATP/ADP ratios were not affected by EA exposure alone, with ratios of 17 ± 2, 15 ± 1
394 and 20 ± 4 following exposure to 100 μM, 200 μM and 300 μM respectively for 3 h. By
395 contrast exposure of cells to H_2O_2, used as a positive control, significantly reduced
396 ATP/ADP ratio from 12 ± 4 to 5 ± 3 after 5 h ($p<0.05$). EA (100 μM) had no influence
397 on H_2O_2-induced reduction in ATP/ADP ratio (5 ± 3 vs. 3 ± 2 nmoles/mg protein,
398 $p>0.05$) (Fig. 8).

399

400 **Changes in intracellular metabonomic profile**

401 Metabonomics was employed to explore the impact of GST inhibitor EA and oxidative
402 stress on global metabolic profile. ^{1}H-NMR spectra acquired from control, EA, H_2O_2
403 and H_2O_2 + EA treated MLE cells. Spectral regions that could be assigned to specific
404 metabolites, that showed visual for evidence of variation in peak intensities, were
405 subject to further analysis. Table 1 shows the fold change of assigned metabolites
406 significantly affected by oxidative stress and EA. Changes in metabolite levels were
407 enhanced after dual treatment with H_2O_2 and EA when compared to treatment with EA
408 or H_2O_2 alone. Metabolites with the most prominent changes in response to treatment
409 with H_2O_2 and EA included: glutamate (4.5-fold increase), threonine (4.2-fold increase),
410 and tyramine (3.5-fold increase). Metabonomics also revealed a number of unassigned
411 metabolites whose levels were altered by H_2O_2 and EA exposure.

412

Genetic manipulation of GSTπ impacts on cell viability in control and stress conditions

To develop a cellular model of GSTM1 null and GSTP1 Ile^{105} polymorphisms, we investigated the impact of specific GST isoform knockdown by RNA interference. The efficiency of siRNA-mediated RNA knockdown was assessed by real time-PCR, 48 h following transfection. Cells transfected with GST μ and π siRNA showed 87 ± 4 % and 90 ± 3 % knockdown of RNA expression respectively, with no significant effect of control scrambled siRNA (Fig. 9A). Protein expression of GST μ and π were evaluated by western blotting 48 and 72 h after transfection. While GSTπ protein expression was abundant in A549 cell line and expressed in whole mouse lung, the expression of this protein was low in MLE cells (Fig. 9 C) and therefore the influence of GST knockdown could not be monitored by western blotting. However, protein bands indicate that GSTμ protein is absent in cells transfected with GSTμ siRNA at 48 and 72 h (Fig 9B).

Oxidative stress was simulated by exposure of cells to H_2O_2 (3 h) or hypoxia (24 h) followed by reoxygenation for 6 h and cell viability was assessed by MTT assay. GSTμ siRNA knockdown had no significant effect on cell viability alone or in response to H_2O_2 or HR. Interestingly GSTπ knockdown resulted in a 16 ± 11 % reduction in viability of control cells (Fig. 10A). Moreover, the viability of GSTπ siRNA-transfected cells was significantly less than cells transfected with scrambled siRNA in the presence of 1 mM H_2O_2 (28 ± 10 % vs. 49 ± 21 %, $p<0.05$) (Fig. 10A). As in previous experimental series, HR did not compromise MLE cell viability in control conditions (scrambled siRNA). However, lack of GSTπ isoform resulted in a 20 ± 18% reduction in viability ($p<0.05$) (Fig. 10B).

EA affects lung mechanics and permeability in the Isolated Perfused Lung model

To begin to explore the physiological role of GST we used an *ex vivo* IPL model and supplemented the perfusate with GST inhibitor EA. Pulmonary mechanics were monitored at 15-min intervals over the 2-h perfusion period. Physiological parameters remained stable and similar to control values for approximately 90 mins of perfusion. However, EA-supplementation resulted in a significant increase in plateau pressure (34 %, $p<0.05$) at 120 mins (Fig. 11A). A similar increase in peak inspiratory pressure (PIP) was also observed (31 %) at 120 mins although this did not reach significance. We observed similar significant increases in pulmonary resistance (29 ± 19, $p<0.05$) and elastance (33 ± 24 %, $p<0.05$) at 120 mins in the presence of EA (Fig. 11B + C).

Lung permeability was assessed via measurement of fluorescence-labelled albumin translocation from blood to alveolar space. BALF protein content was measured as an additional index of pulmonary oedema. EA gave a 3-fold rise in the BALF:perfusate ratio, an index of epithelial barrier permeability (0.017 ± 0.009 vs. 0.052 ± 0.016, $p<0.05$) (Fig. 11D), and a 6-fold increase in BALF protein levels (0.7 ± 0.4 vs. 4.3 ± 2.4 mg/ml, $p<0.05$) (Fig. 11E).

DISCUSSION

There is increasing evidence from clinical literature that GST polymorphism and altered GST expression may impact on lung oxidative stress and outcomes of lung injury (7, 25, 32). Experimental data shows that exogenous supplementation of recombinant GST enzymes impacts positively on oxidative events and inflammation (23) but the role of endogenous GSTs is less well known. The primary objective of this study was to explore the influence of attenuated endogenous GST activity in lung epithelial cells on cellular responses relevant to the setting of ischemia and reperfusion injury.

In the present study, we demonstrated that both pharmacological inhibition of endogenous GSTs and genetic knockdown of GST isoforms affected epithelial cell injury. Firstly, two well recognised GST inhibitors, EA and CA (28, 29, 49) compromised cell viability in a concentration- and time-dependent manner. Concentrations inducing such cytotoxicity were comparable to those inhibiting both pure GST and endogenous GST activity in MLE cell lysates. While GST inhibitor-induced cell death was unexpected, such a response is not unprecedented as similar observations were made in colon cancer cell lines and human leukaemia cell lines (1, 37). In addition to causing cell injury themselves, subtoxic concentrations of EA and CA potentiated cytotoxicity induced by chemical stressors H_2O_2 and tBH. While MLE cells tolerated long periods of hypoxia and subsequent reoxygenation, injury was unmasked in the presence of subtoxic concentrations of the GST inhibitor.

Genetic attenuation of GSTπ following siRNA transfection resulted in a decrease in viability of non-stressed cells and rendered cells more susceptible to H_2O_2-induced injury and HR conditions. Although the magnitude of this response was less than those

with pharmacological inhibition, this is remarkable considering the redundancy of intracellular GST enzymes.

While our experiments may suggest a more specific involvement of GSTπ, as knockdown of GSTμ did not have similar effects in MLE cells, these results should be interpreted with caution. Firstly, we only targeted one GSTM gene and other genes in this class could have compensated for such effect. Secondly, the abundance of GSTπ in MLE cells was lower than GSTμ and the effect of knockdown on cell viability, therefore, could have been related to protein abundance rather than specific activity. Nevertheless, our results suggest that endogenous GSTπ could be important for normal homeostasis and viability of lung epithelial cells and in the intrinsic survival mechanisms of these cells under oxidative stress conditions and hypoxia/reoxygenation.

Apoptosis of lung epithelial cells represents a potentially important mechanism contributing to the development of acute lung injury in various settings. Two previous studies evaluated the role of apoptosis in EA-induced cell death in cancer cell lines and reported conflicting results (1, 37). By using flow cytometry, we observed a slight increase in the percentage of purely apoptotic cells following EA exposure, however, the majority of cells exhibited both Annexin V and PI fluorescence, representing a late apoptotic/necrotic cell death. To further define the role of classical apoptotic events in EA-induced cell death, we investigated the involvement of caspases. EA-induced cell death was not affected by the caspase inhibitor, which in turn attenuated the response to another death stimulus (TNF in the presence of cycloheximide). Thus we concluded that EA-induced cytotoxicity is unlikely to be driven by a classical caspase-mediated

apoptotic pathway. However, we cannot exclude the involvement of a caspase-independent apoptotic cascade, such as that mediated by apoptosis-inducing factor.

We have considered the possibility that the GST inhibitors increased intracellular oxidative stress, either through inhibition of GST leading to an accumulation of intracellular ROS, or through direct activation of ROS- producing pathways. In previous studies, ROS accumulation has been implicated in EA-induced cell death (37), thus we investigated the influence of EA on various aspects of oxidative stress using complementary approaches. EA caused a significant increase in both DHR123 (2-fold) and DCF (30-fold) fluorescence in MLE cells. Although the exact mechanism responsible for the increased fluorescence and differential response with these probes remains unknown, the data provides evidence linking EA exposure to intracellular oxidative reactions. While the limitation of DCF in detecting intracellular H_2O_2 is well established (15, 40) it is possible that EA-induced redox events may involve H_2O_2-triggered downstream oxidative reactions. Indeed, one previous study concluded that EA-induced cytotoxicity was mediated by H_2O_2 (37). In our experiments, however, H_2O_2 caused a dissimilar pattern of DCF/DHR 123 increase when compared to EA, and EA-induced cell injury was not prevented by PEG catalase or potentiated by endogenous catalase inhibition. A limitation of these experiments is that intracellular catalase activity was not measured. Furthermore, the application of DETC may not have been specific as it affects all copper-containing molecules, not just CuZnSOD and it would not be expected to affect MnSOD. The DHR response indicates the possible involvement of reactive nitrogen species, however, L-NAME had no effect on EA responses. Therefore, we do not think that either H_2O_2 or nitric oxide play a significant role in GST inhibitor-induced intracellular oxidative stress and cell death.

To further substantiate the role of redox events we determined global intracellular redox state by measuring the prominent intracellular NAD^+/NADH redox couples. Cellular and mitochondrial redox homeostasis is intricately connected and regulated by several redox-sensitive couples including glutathione/glutathione disulphide and NAD^+/NADH (2). We have shown the ability of EA to alter NAD^+/NADH ratio and a tendency to potentiate H_2O_2-induced redox changes. Furthermore, although low concentrations of EA did not cause protein carbonyl formation, EA significantly enhanced this reaction by H_2O_2. Taken together, these independent experiments suggest an association between EA-induced intracellular oxidative stress and cellular injury.

Interestingly, pre-incubation of cells with NAC prevented both EA- and CA-induced increase in ROS, as well as the resulting cell death. There may be several explanations for this phenomenon. Firstly, NAC may interfere with the GST inhibitors themselves in the extracellular space. Although we cannot exclude such possibility with certainty, the fact that NAC inhibited the actions of two chemically distinct pharmacological GST inhibitors makes this scenario less likely. Moreover, Aizawa et al. also concluded that the influence of NAC on EA-induced responses in their experiments was not related to direct interaction with EA (1). The second explanation is that NAC could augment intracellular antioxidant capacity by entering the cells and may help scavenge reactive species responsible for fluorescent dye oxidation and cellular injury. Thirdly, NAC as a precursor of glutathione may augment or replenish depleted glutathione levels. Indeed, DCF fluorescence is modulated by intracellular glutathione and glutathione is one of the major defence mechanisms guarding against intracellular oxidative stress. Finally, the effects of NAC may be unrelated to its antioxidant properties as it is a very broad-spectrum reducing agent.

557 The effects of GST inhibitors on mitochondrial energetics and its influence on cell injury
558 and necrosis were considered. As ATP/ADP ratio remained unchanged even by high
559 concentrations of EA, we have concluded that mitochondrial dysfunction is an unlikely
560 mediator of EA-induced cell death. Metabonomics profiling has provided a preliminary
561 insight into the metabolic pathways affected in cells exposed to oxidant stress and/or
562 GST inhibition. Initial multivariate analysis of acquired NMR spectral dataset separated
563 treatment groups suggesting that both oxidative stress and GST inhibition produce time-
564 and treatment-specific changes in the global intracellular metabolome. Targeted spectral
565 analysis identified specific changes in cellular metabolites in response to defined
566 oxidative stress by H_2O_2 and many of these have close links to energy metabolism and
567 cellular redox status and adaptation
568
569 These metabolites have been previously reported to be associated with various ischemic,
570 inflammatory and oxidative conditions. For instance, glutamate acts a neurotransmitter
571 in various oxidative stress-related neurodegenerative conditions and has been linked to
572 the development of lung injury in animal models of hyperoxia and sepsis (10, 30, 36). In
573 addition, threonine levels increase in response to hypoxic injury (42) and elevated levels
574 of tyramine have been reported following myocardial infarction, acute cystitis and
575 smoking (22, 27, 43). Further experimental work and bioinformatics analysis is required
576 to determine the precise role of these metabolites in ischemia, oxidative stress and
577 inflammation.
578
579 It is clear that multiple oxidative stress-induced changes were modified by co-treatment
580 with the GST inhibitor. By detecting differences in a number of unassigned peaks of the
581 metabonomic spectra, our findings also reveal potentially novel metabolic signatures of
582 oxidative stress and GST inhibition.

In addition to the in vitro studies, experiments were performed to explore the physiological consequences of GST inhibition. A mouse *ex vivo* lung perfusion model documented the direct pulmonary influence of EA supplementation. EA caused time-dependent changes in multiple aspects of physiology, in particular, increased respiratory elastance, resistance, and pulmonary permeability. The experimental conditions did not affect perfusion pressure. These preliminary findings suggest that EA caused a degree of specific injury to lung compartments, most likely microvascular and epithelial permeability.

Despite the strengths of our investigation covering multiple aspects of endogenous GST-related oxidative stress, cellular energetics and metabolism using complementary pharmacological and genetic approaches, and expanding the *in vitro* findings to *in vivo* physiology, the study has limitations. Firstly, our investigation focuses primarily on epithelial cells. We however realise that hypoxia/reperfusion injury requires damage to both epithelial and endothelial linings of the lung and the endothelium must be damaged for the increase in permeability and changes in lung mechanics to occur. There is published evidence regarding the roles for GST enzymes in modulating cellular responses of vascular endothelial cells and signaling pathways during oxidative stress (46, 48). Moreover, we show that the cytotoxic effect of EA is not restricted to epithelial cells but the phenomenon is also observed in mouse endothelial cells. Our IPL data also demonstrate selective influence of EA on endothelial function as pulmonary artery pressure, hence pulmonary vascular resistance and vasoactivity was not altered but microvascular permeability was increased. This phenomenon may be related to published evidence of GST regulating tight junction composition and organisation (46).

Another limitation relates to using fluorescence markers of oxidative stress which is liable to well-known limitations and potential artefacts (15, 40). We realise that fluorescent dyes only provide general information on the ability of the cells to oxidise the dyes and that the exact mechanism of these phenomena remain to be fully elucidated. However, we did not rely completely on these probes but assessed additional aspects of oxidative stress and multiple pharmacological strategies.

A further limitation is that most of the GST inhibition experiments used pharmacological tools that have the potential for off-target effects. Nevertheless, we obtained similar data using chemically distinct inhibitors and complemented the pharmacology with genetic approaches. Finally our metabonomics and *in vivo* studies are preliminary in nature. Nevertheless, the metabolic profiling experiments have provided us with the first description of oxidative stress metabolome in lung epithelial cells. Furthermore, the physiological experiments clearly documented promising aspects of lung mechanics and permeability where endogenous GST may be essential. These may be helpful in the design of *in vivo* experiments for future studies, perhaps using GST conditional cell-specific knockout mice.

This work and other published literature should underpin more preclinical work with animal models and towards new approaches to address lung injury in humans. The recognition that GST genes are consistently altered in various animal models of lung injury and are among the top genes that accurately predict development of primary graft failure in lung transplant donors (19) certainly points towards these promising directions. Our pilot genetic data may also highlight a role of specific GST gene polymorphism in the development of perioperative myocardial infarction (20) thus extending the paradigm beyond lung ischemia reperfusion injury.

SUMMARY

In summary, we have demonstrated that alteration of GST activity disturbs the intracellular redox status, most likely through accumulation of secondary oxidative stress products. Furthermore, our findings suggest that GST inhibitors or specific attenuation of GSTπ enhance H_2O_2-induced cell injury due to increased oxidative stress and subsequent metabolic derangements. The totality of evidence gathered in these studies demonstrates that GST activity is potentially involved in the survival of cultured lung epithelial cells under stress conditions. Based on our pilot physiological data, endogenous GST may play an important role in lung microvascular and epithelial regulation of permeability and lung mechanics and may have important clinical implications.

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652 **Table 1.** Fold change of selected metabolites significantly affected by Ethacrynic acid
653 (100 μM) and oxidative stress (0.8 mM H_2O_2). Cells were exposed to either or both of
654 these treatments over a 5 h period (N=3)

	EA	**H_2O_2**	**H_2O_2 + EA**	***P*†**
Threonine	2.0	2.4	4.2	0.016
Glutamate	1.6	2.1	4.5	0.016
Tyramine	1.5	1.9	3.5	0.019
Phenylalanine	1.5	2.0	3.2	0.022
Lactate	1.4	1.6	2.2	0.025
Pyruvate	1.4	1.1	1.5	0.022
Ox-glutathione	1.3	1.2	1.6	0.025
Alanine	1.2	1.2	1.4	0.025
Aspartate	1.1	1.2	1.6	0.034
Free choline	1.1	1.3	1.5	0.016
Glycerophosphocholine	1.3	1.0	1.8	0.024

† Kruskal-Wallis test. Multiple comparisons were controlled for using the Benjamini–Hochberg procedure, with accepted false discovery rate 0.05.

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656

657

FIGURE LEGENDS

Figure 1. Influence of increasing concentrations of GST inhibitors on MLE cell viability. Viability was assessed by MTT assay following (A) 5 h EA exposure and (B) 24 h treatment with CA. N=4-6; *p<0.05 vs. untreated control.

Figure 2. Apoptotic/necrotic cells determined by Annexin-V/PI staining and flow cytometry. Cells were treated with 300 μM EA for the indicated times and untreated cells were used as a negative control. A representative FSC/SSC gating strategy is illustrated in the panel to exclude cell debris from the analysis. The gated cell population was then acquired for Ann V and PI staining. Viable: Ann V- and PI-negative. Apoptotic: Ann V-positive and PI-negative. Necrotic: PI-positive. Apop/Nec: Ann V- and PI-positive.

Figure 3. Influence of caspase inhibitor zVAD on stress-induced MLE cell death. MLE cells were exposed to stresses (**A**) EA- and (**B**) tumour necrosis factor alpha (TNFα)/cycloheximide for 5 and 24 h, respectively. N=3-6; ***** p<0.05 vs. vehicle control (i.e. in the absence of zVAD).

Figure 4. Impact of GST inhibition on MLE cell response to oxidative stress conditions. Influence of chemical stressors hydrogen peroxide (H_2O_2) (**A** + **B**) and tert-butyl hydroperoxide (tBH) (**C**) on cell viability, determined by MTT assay, in the absence (▲) and presence of GST inhibitors EA (○) and CA (●). (**D**) Exposure of cells to 24 h of either normoxic or hypoxia (24 h) followed by reoxygenation (6 h) (Hypoxia/reoxygenation - HR) in the presence of indicated concentrations of EA. N=5;

683 *p<0.05 vs. vehicle controls (without GST inhibitors); †p<0.05 vs. appropriate
684 normoxic control.
685
686 **Figure 5.** Influence of GST inhibitor EA on intracellular oxidative stress measured by
687 fluorescence of redox sensistive dyes (**A**) Dihydrorhodamine 123 (DHR 123) and (**B**)
688 Dichlorofluorescein (DCF) fluorescence, following 1 h incubation with indicated
689 concentrations of EA. N=5. Intracellular redox status was assessed by NAD^+/ NADH
690 (by measuring ADPR, a breakdown product of NADH under acid precipitation
691 conditions) ratio following (**C**) increasing concentrations of EA (over 1 h) and (**D**) EA
692 (100 μM) treatment +/- 0.8 mM H_2O_2 stress over 3 h. N=3. (**E**) Influence of EA (100
693 μM) and/or H_2O_2 (0.8 mM) on protein carbonyl levels after 3 h treatment. N=3; *p<0.05
694 vs. untreated control, $^{\Delta}$ p<0.05 vs. cells exposed to H_2O_2.
695
696 **Figure 6.** Influence of catalase on (A) EA- and (B) H_2O_2-induced MLE cell death.
697 Cells were treated for 5 h and catalase groups are represented by the dotted line. N=4-6.
698 Influence of catalase inhibitor aminotriazole (ATZ) on ethacrynic acid (EA)-induced
699 cytotoxicity is depicted in panel C. Cells were treated for 5 h and ATZ is represented by
700 dotted line. N=4.
701
702
703 **Figure 7.** Influence of N-acetyl cysteine (NAC) on ethacrynic acid (EA)-induced
704 increase in fluorescence of 'redox sensitive dyes (A) dihydrorhodamine 123 (DHR123)
705 and (B) dichlorofluorescein (DCF) over 1 h. N=4; *p<0.05 vs. vehicle control (in the
706 absence of NAC).

Panel C and D depicts the influence of (NAC) (▲) on (C) EA- (○) and (D) CA- (●) induced cell death, over 5 and 24 h respectively, assessed by MTT assay. N=6. *p<0.05 vs. vehicle control (in the absence of catalase or NAC).

Figure 8. Mitochondrial energetics was assessed by ATP/ADP ratio following (A) increasing concentrations of ethacrynic acid (EA) over 3 h and (B) EA (100 μM) treatment and/or H_2O_2 (0.8 mM) over 5 h. N=3; *p<0.05 vs. untreated control.

Figure 9. Efficiency of GST μ and π siRNA and impact of GST isoform knockdown on MLE cells exposed to stress. (**A**) GSTμ RNA expression measured by RT-PCR 48 h after cell transfection with scrambled, μ and π siRNA. N=4; *p<0.05 vs. scrambled control. (**B**) A representative blot (n=3) of GSTμ protein expression assessed by western blotting 48 and 72 h after cells were non-transfected (NT), or transfected with scrambled or GSTμ siRNA. (C) Western blot analysis of GST μ and π protein expression in MLE and A549 cell lysates and whole mouse lung homogenate (as additional controls) N=2.

Figure 10. Impact of GST isoform knockdown on MLE cells exposed to stress. The influence of GST μ and π attenuation on cellular response to chemical stress H_2O_2 at the indicated concentrations (over 3 h) is shown in Panel C and to 24 h hypoxia and 6 h reoxygenation (HR) in Panel D. N=4-6; *p<0.05 vs. control cells transfected with scrambled siRNA.

Figure 11. Effect of EA-supplemented perfusion on pulmonary mechanics of mouse IPL (over 120 mins). (**A**) plateau pressure, (**B**) pulmonary resistance and (**C**) pulmonary elastance were all monitored at 15-min intervals in murine lungs perfused with control (▲) or EA (100 μM)-supplemented (○) perfusate. Influence of EA-supplemented perfusate on (**D**) endothelial and epithelial barrier permeability and (**E**) BALF protein content, N=3; *$p<0.05$ vs. vehicle control.

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Figure 1

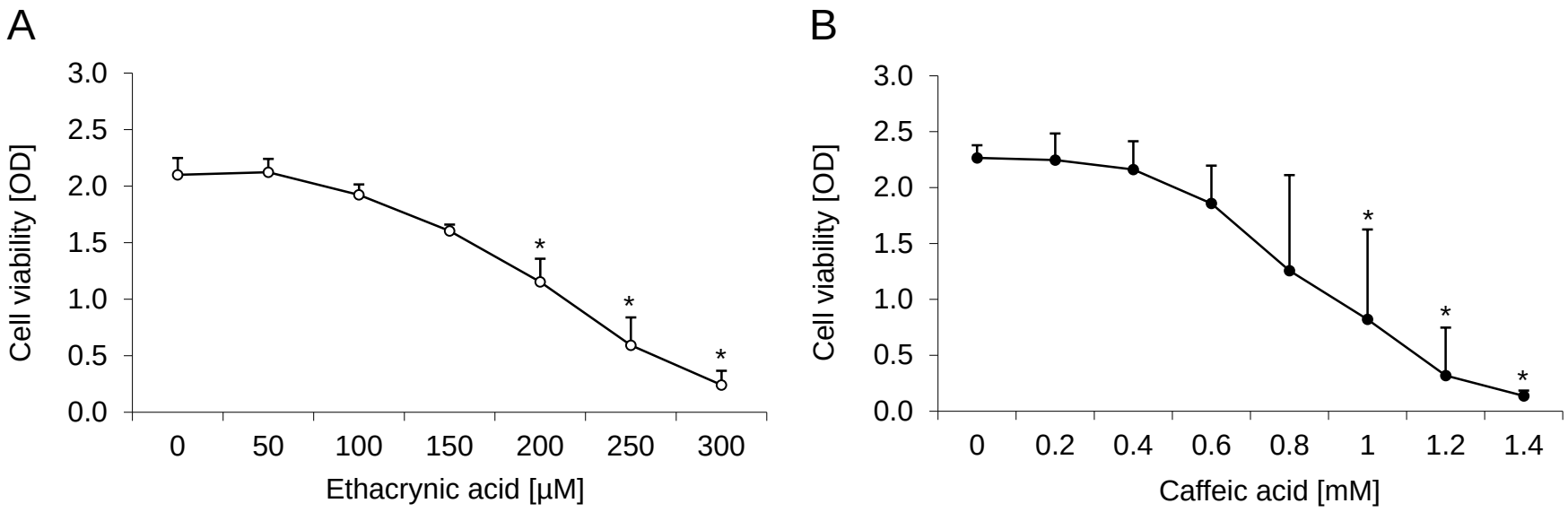
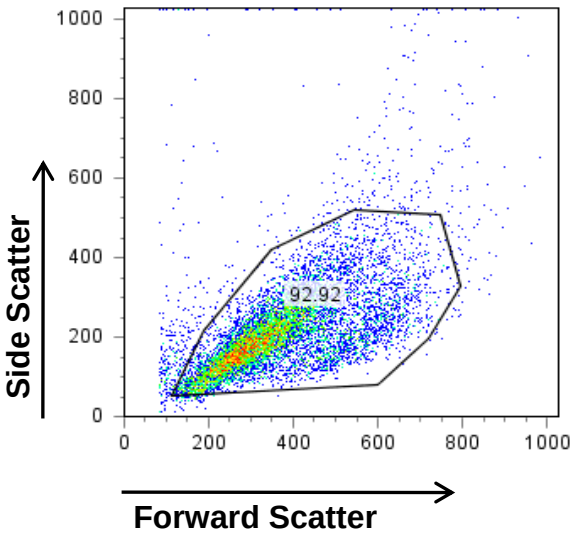

A
Cell viability [OD]
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0
50
100
150
200
250
300
Ethacrynic acid [µM]
*
*
*
B
Cell viability [OD]
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0
0.2
0.4
0.6
0.8
1
1.2
1.4
Caffeic acid [mM]
*
*
*

Figure 2


Side Scatter
Forward Scatter
92.92

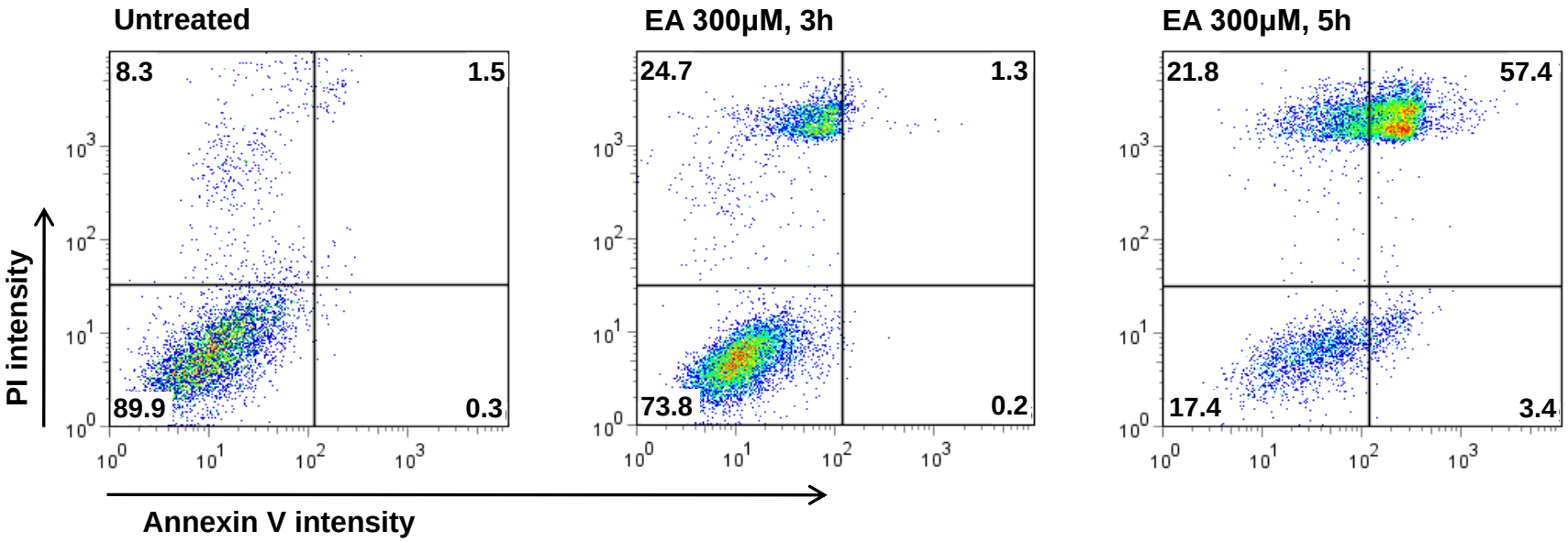

Untreated
EA 300μM, 3h
EA 300μM, 5h
8.3
1.5
89.9
0.3
24.7
1.3
73.8
0.2
21.8
57.4
17.4
3.4
PI intensity
Annexin V intensity

Figure 3

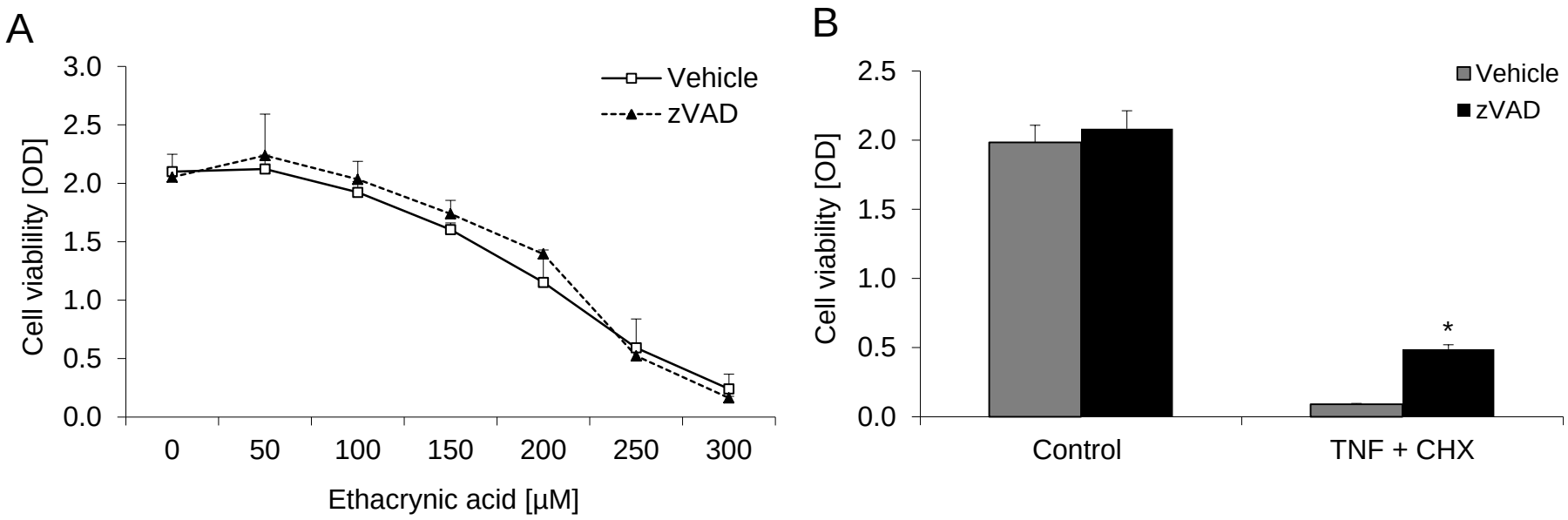

A
Cell viability [OD]
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0
50
100
150
200
250
300
Ethacrynic acid [µM]
Vehicle
zVAD
B
Cell viability [OD]
2.5
2.0
1.5
1.0
0.5
0.0
*
Control
TNF + CHX
Vehicle
zVAD

Figure 4

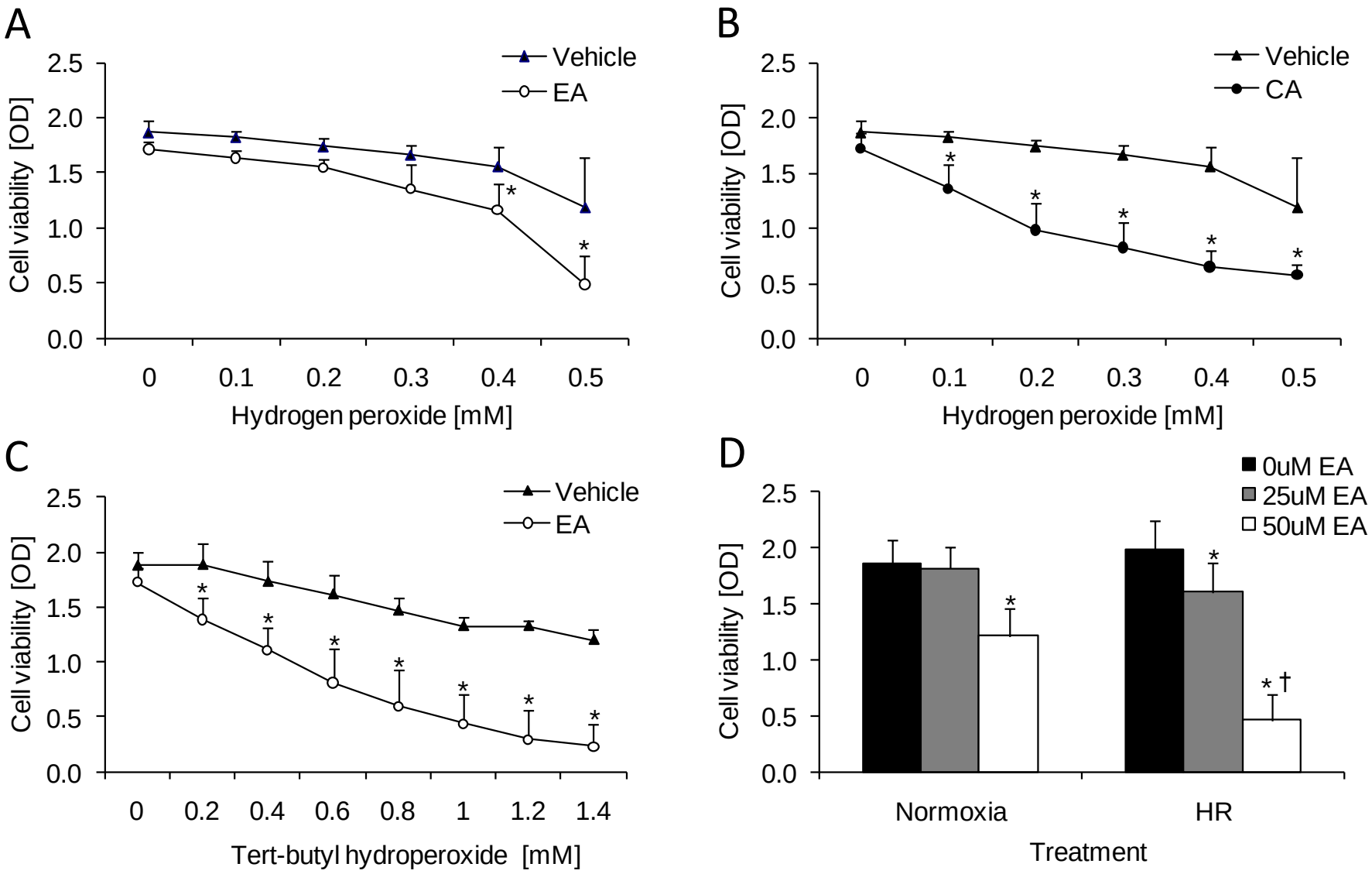

A
Vehicle
EA
Cell viability [OD]
2.5
2.0
1.5
1.0
0.5
0.0
0
0.1
0.2
0.3
0.4
0.5
Hydrogen peroxide [mM]
B
Vehicle
CA
Cell viability [OD]
Hydrogen peroxide [mM]
C
Vehicle
EA
Cell viability [OD]
0
0.2
0.4
0.6
0.8
1
1.2
1.4
Tert-butyl hydroperoxide [mM]
D
0uM EA
25uM EA
50uM EA
Cell viability [OD]
Normoxia
HR
Treatment

Figure 5

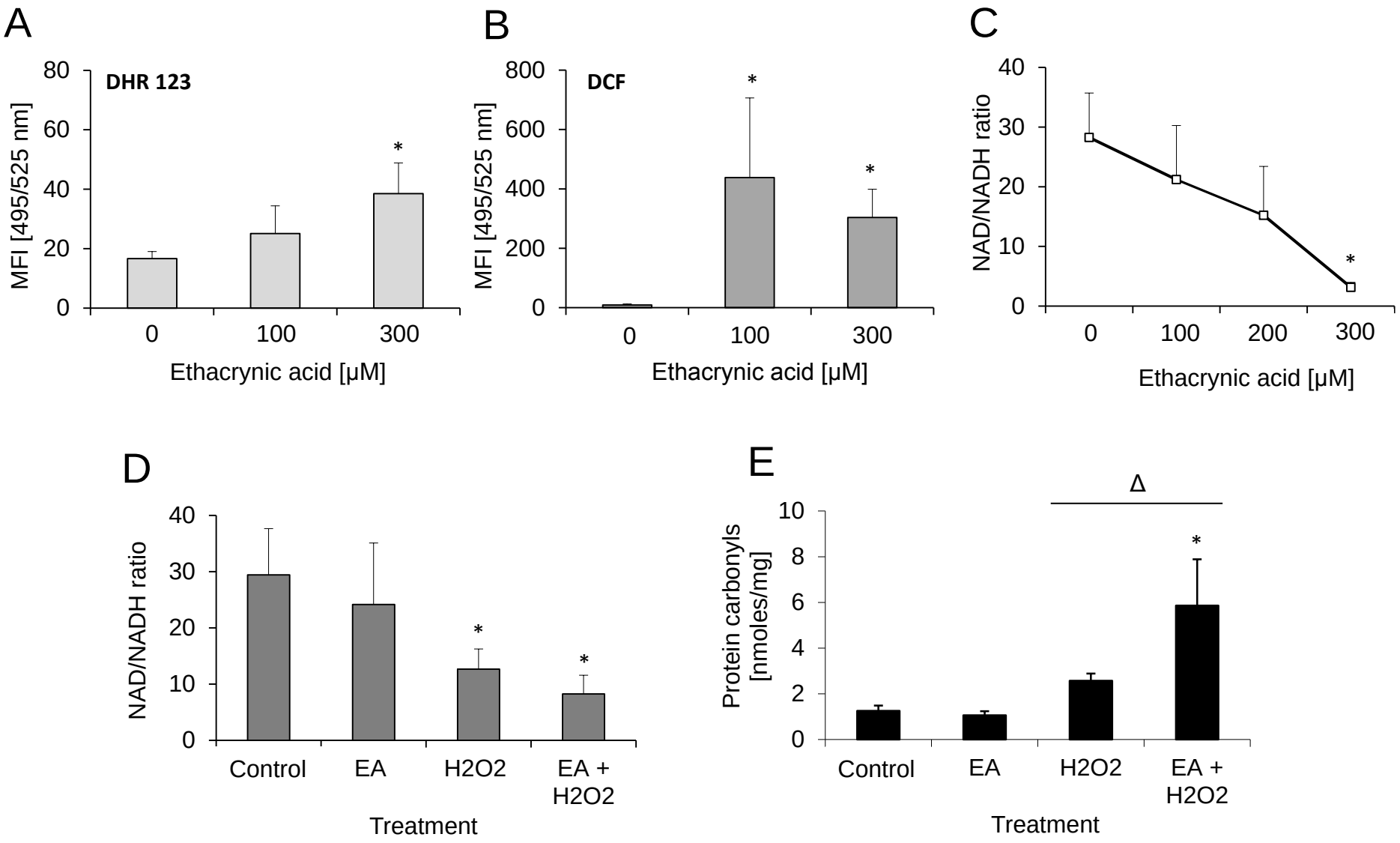

A
DHR 123
MFI [495/525 nm]
80
60
40
20
0
0
100
300
Ethacrynic acid [μM]
*
B
DCF
MFI [495/525 nm]
800
600
400
200
0
0
100
300
Ethacrynic acid [μM]
*
*
C
NAD/NADH ratio
40
30
20
10
0
0
100
200
300
Ethacrynic acid [μM]
*
D
NAD/NADH ratio
40
30
20
10
0
Control
EA
H2O2
EA + H2O2
Treatment
*
*
E
Δ
Protein carbonyls [nmoles/mg]
10
8
6
4
2
0
Control
EA
H2O2
EA + H2O2
Treatment
*

Figure 6

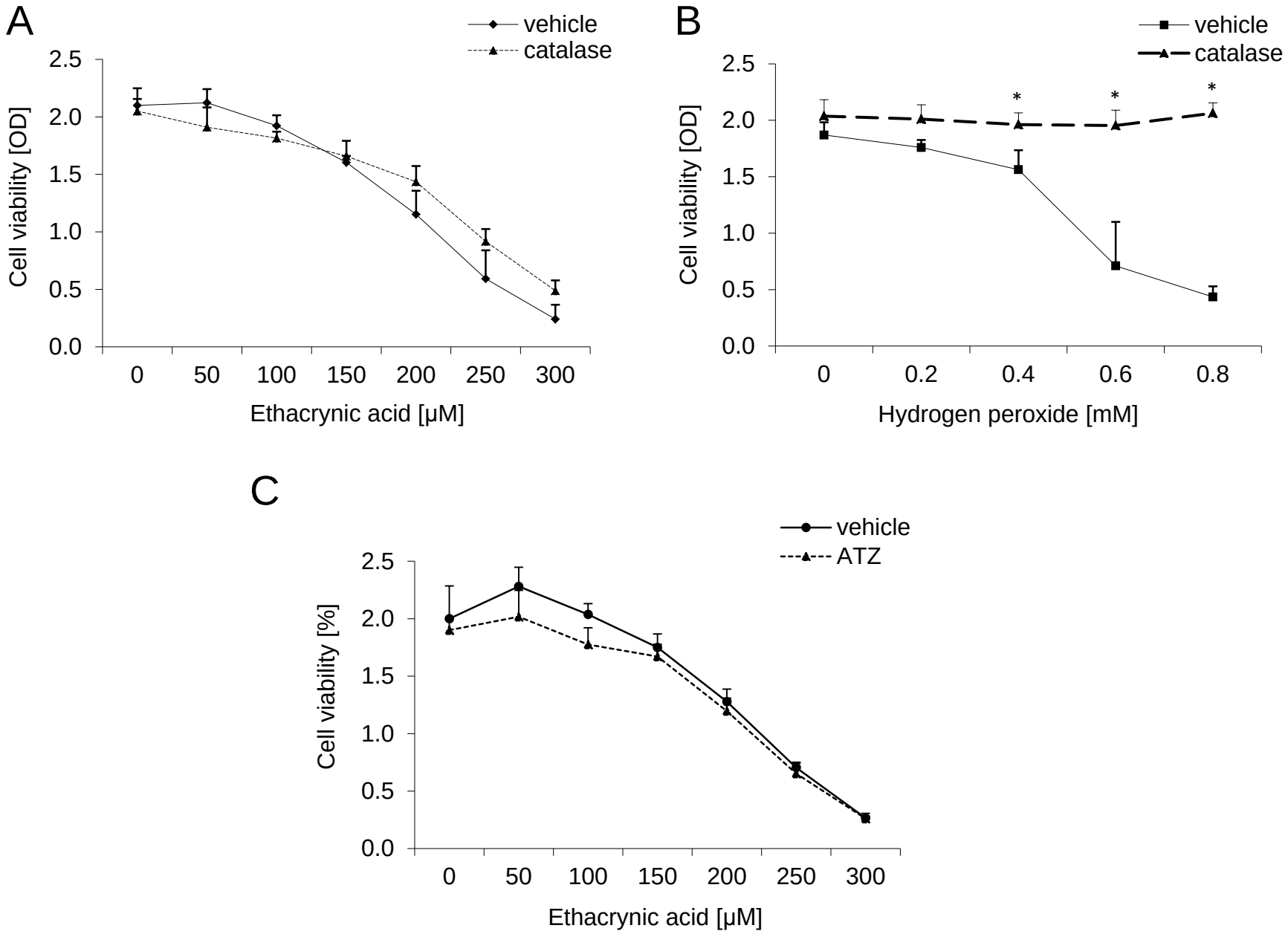

A
vehicle
catalase
Cell viability [OD]
2.5
2.0
1.5
1.0
0.5
0.0
0
50
100
150
200
250
300
Ethacrynic acid [μM]
B
vehicle
catalase
Cell viability [OD]
2.5
2.0
1.5
1.0
0.5
0.0
*
*
*
0
0.2
0.4
0.6
0.8
Hydrogen peroxide [mM]
C
vehicle
ATZ
Cell viability [%]
2.5
2.0
1.5
1.0
0.5
0.0
0
50
100
150
200
250
300
Ethacrynic acid [μM]

Figure 7

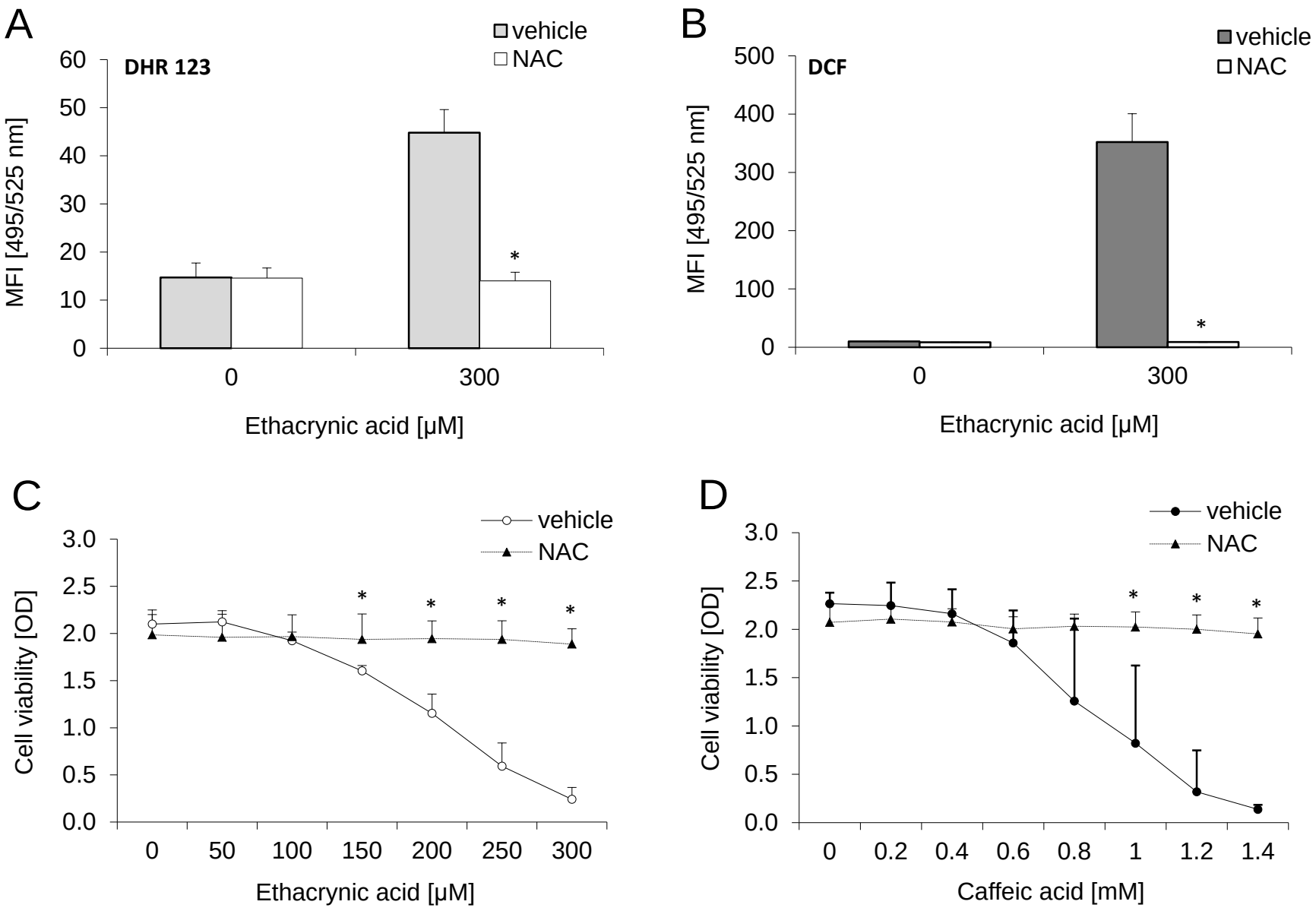
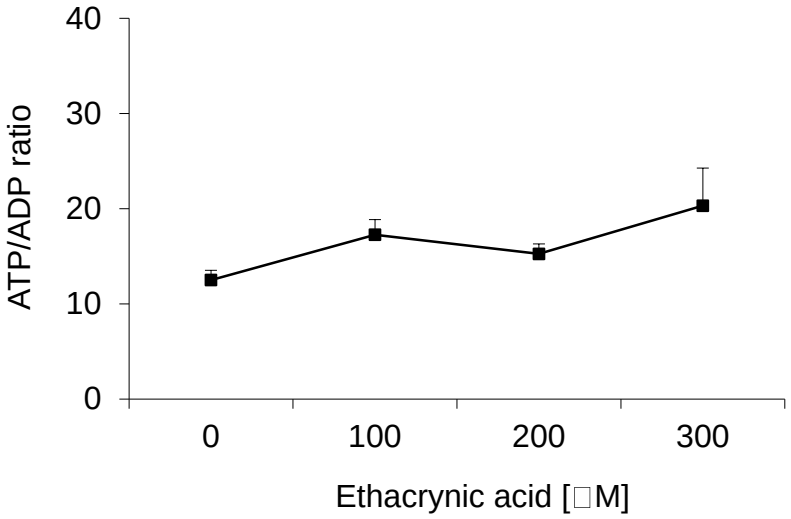

A
DHR 123
vehicle
NAC
MFI [495/525 nm]
60
50
40
30
20
10
0
0
300
Ethacrynic acid [μM]
*
B
DCF
vehicle
NAC
MFI [495/525 nm]
500
400
300
200
100
0
0
300
Ethacrynic acid [μM]
*
C
vehicle
NAC
Cell viability [OD]
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0
50
100
150
200
250
300
Ethacrynic acid [μM]
*
D
vehicle
NAC
Cell viability [OD]
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0
0.2
0.4
0.6
0.8
1
1.2
1.4
Caffeic acid [mM]
*

Figure 8

A


40
30
20
10
0
ATP/ADP ratio
0
100
200
300
Ethacrynic acid [□M]

B

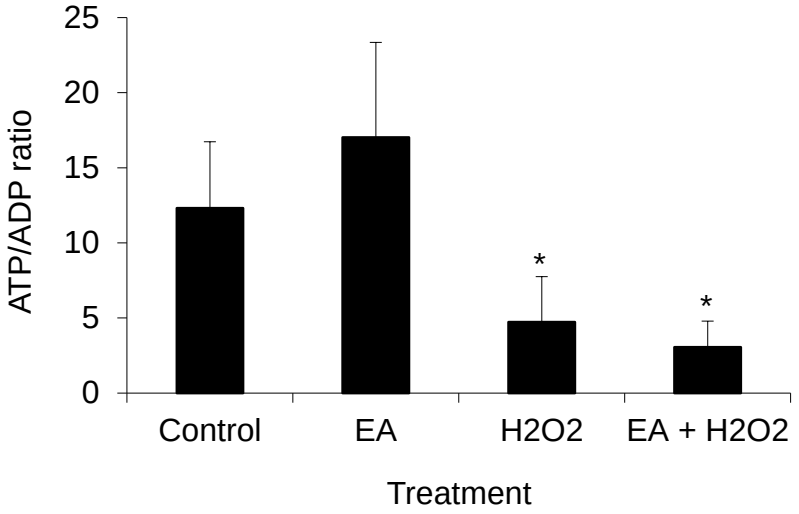
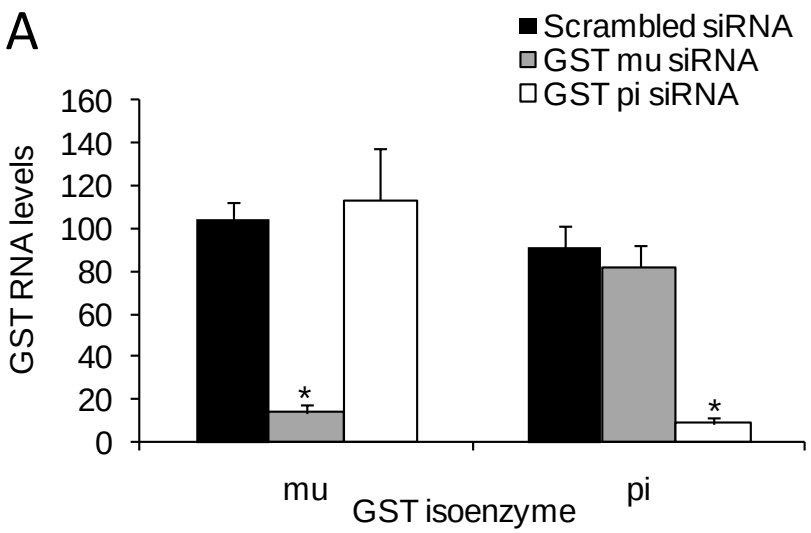
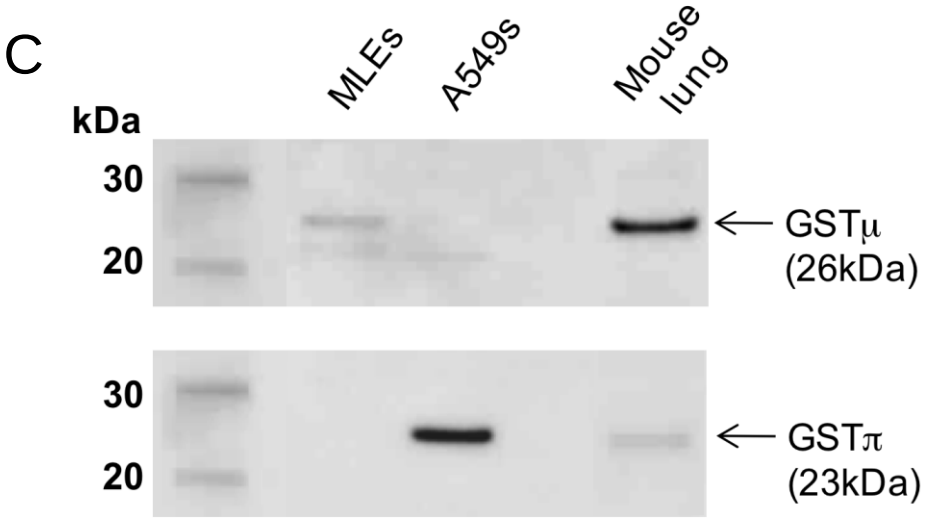

25
20
15
10
5
0
ATP/ADP ratio
*
*
Control
EA
H2O2
EA + H2O2
Treatment

Figure 9


A
Scrambled siRNA
GST mu siRNA
GST pi siRNA
GST RNA levels
160
140
120
100
80
60
40
20
0
*
*
mu
pi
GST isoenzyme


C
MLEs
A549s
Mouse lung
kDa
30
20
GSTμ (26kDa)
30
20
GSTπ (23kDa)

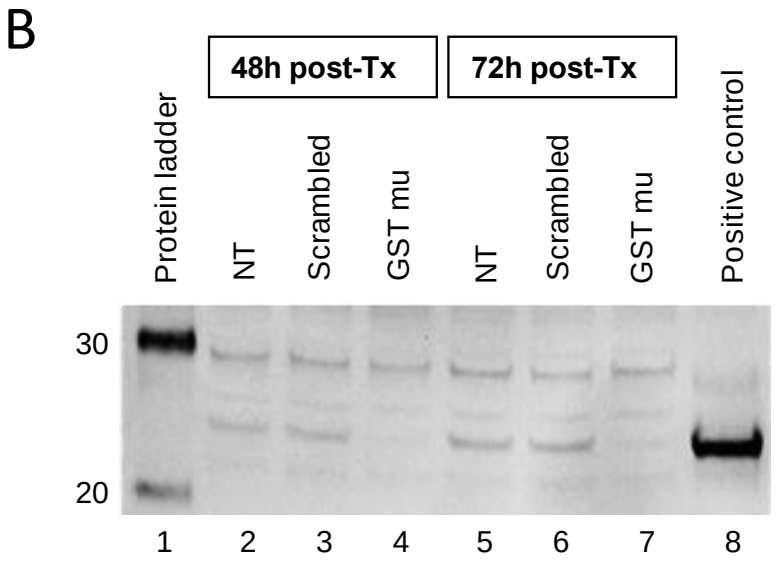

B
48h post-Tx
72h post-Tx
Protein ladder
NT
Scrambled
GST mu
NT
Scrambled
GST mu
Positive control
30
20
1
2
3
4
5
6
7
8

Figure 10

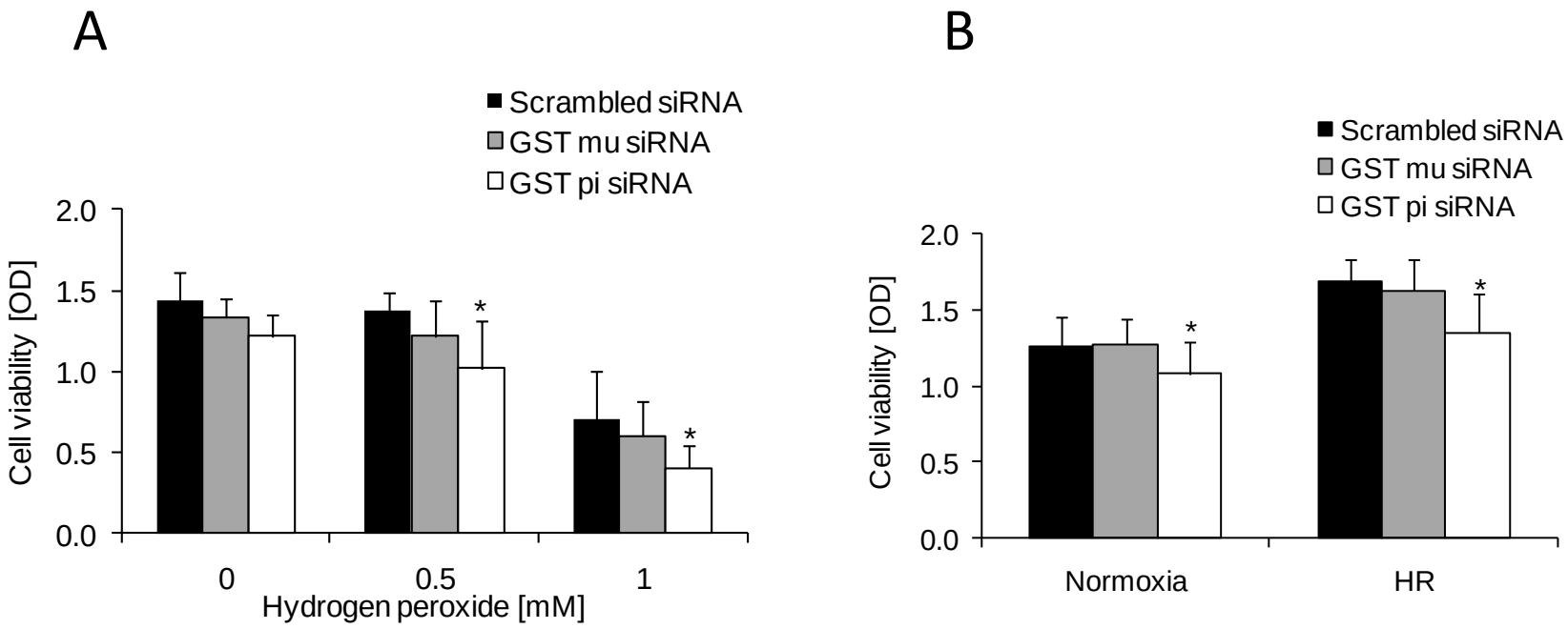
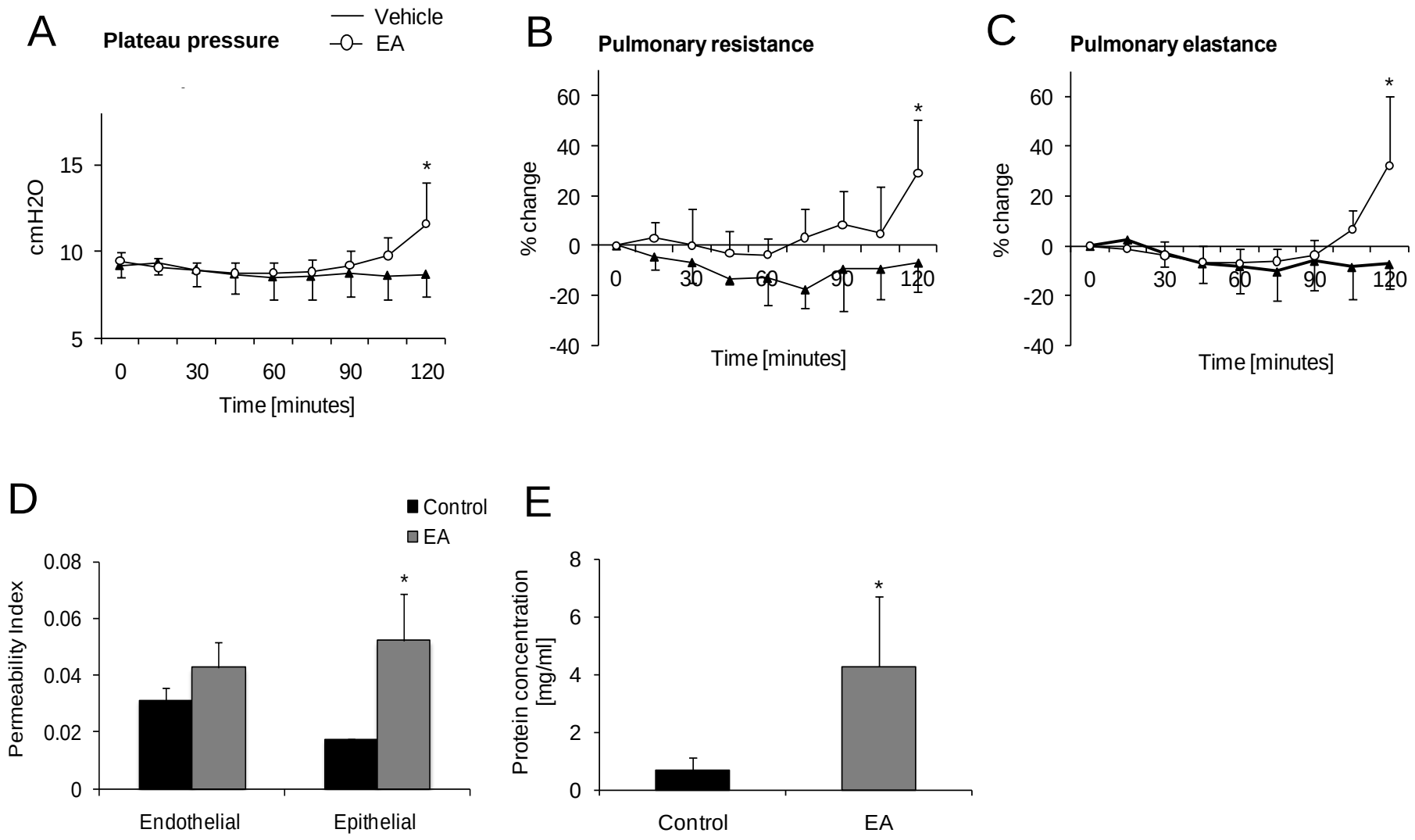

A
Scrambled siRNA
GST mu siRNA
GST pi siRNA
Cell viability [OD]
2.0
1.5
1.0
0.5
0.0
*
*
0
0.5
1
Hydrogen peroxide [mM]
B
Scrambled siRNA
GST mu siRNA
GST pi siRNA
Cell viability [OD]
2.0
1.5
1.0
0.5
0.0
*
*
Normoxia
HR

Figure 11


A
Plateau pressure
Vehicle
EA
cmH2O
15
10
5
0
30
60
90
120
Time [minutes]
*
B
Pulmonary resistance
% change
60
40
20
0
-20
-40
0
30
60
90
120
Time [minutes]
*
C
Pulmonary elastance
% change
60
40
20
0
-20
-40
0
30
60
90
120
Time [minutes]
*
D
Control
EA
Permeability Index
0.08
0.06
0.04
0.02
0
Endothelial
Epithelial
*
E
Protein concentration [mg/ml]
8
6
4
2
0
Control
EA
*