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Gsta4 Null Mouse Embryonic Fibroblasts Exhibit Enhanced Sensitivity to Oxidants: Role of 4-Hydroxynonenal in Oxidant Toxicity*

Kevin E. McElhanon[1,2], **Chhanda Bose**[2,3], **Rajendra Sharma**[1,2], **Liping Wu**[1,2], **Yogesh C. Awasthi**[4], and **Sharda P. Singh**[1,2,#]

[1]Department of Pharmacology and Toxicology, University of Arkansas for Medical Sciences, Little Rock, USA

[2]Central Arkansas Veterans Healthcare System, Little Rock, USA

[3]Department of Internal Medicine, Nephrology Division, University of Arkansas for Medical Sciences, Little Rock, USA

[4]Department of Molecular Biology and Immunology, University of North Texas Health Science Center, Fort Worth, USA

Abstract

The alpha class glutathione s-transferase (GST) isozyme GSTA4–4 (EC2.5.1.18) exhibits high catalytic efficiency to-wards 4-hydroxynon-2-enal (4-HNE), a major end product of oxidative stress induced lipid peroxidation. Exposure of cells and tissues to heat, radiation, and chemicals has been shown to induce oxidative stress resulting in elevated concentrations of 4-HNE that can be detrimental to cell survival. Alternatively, at physiological levels 4-HNE acts as a signaling molecule conveying the occurrence of oxidative events initiating the activation of adaptive pathways. To examine the impact of oxidative/electrophilic stress in a model with impaired 4-HNE metabolizing capability, we disrupted the *Gsta*4 gene that encodes GSTA4–4 in mice. The effect of electrophile and oxidants on embryonic fibroblasts (MEF) isolated from wild type (WT) and *Gsta*4 null mice were examined. Results indicate that in the absence of GSTA4–4, oxidant-induced toxicity is potentiated and correlates with elevated accumulation of 4-HNE adducts and DNA damage. Treatment of *Gsta*4 null MEF with 1,1,4-tris(acetyloxy)-2(E)-nonene [4-HNE$(Ac)_3$], a pro-drug form of 4-HNE, resulted in the activation and phosphorylation of the c-jun-N-terminal kinase (JNK), extracellular-signal-regulated kinases (ERK 1/2) and p38 mitogen activated protein kinases (p38 MAPK) accompanied by enhanced cleavage of caspase-3. Interestingly, when recombinant mammalian or invertebrate GSTs were delivered to *Gsta*4 null MEF, activation of stress-related kinases in 4-HNE$(Ac)_3$ treated *Gsta*4 null MEF were inversely correlated with the catalytic efficiency of delivered GSTs towards 4-HNE. Our data suggest that GSTA4–4 plays a major role in protecting cells from the toxic effects of oxidant chemicals by attenuating the accumulation of 4-HNE.

Keywords

Glutathione Transferase; Protein Adducts; Lipid Peroxidation; Oxidants; Stress Kinases

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#Corresponding author. SinghShardaP@uams.edu.

1. Introduction

Lipid aldehydes generated during lipid peroxidation (LPO) are highly reactive species capable of electrophilic attack on DNA and proteins [1–4]. 4-HNE, one of the major end products of LPO, causes cytotoxicity and genotoxicity by inducing necrosis and pro-apoptotic signaling through multiple pathways at supraphysiological concentrations [5–7]. 4-HNE is also involved in regulation of gene expression and cell cycle signaling in a concentration dependent manner [8–11]. We and others [12,13] have shown that 4-HNE causes activation and phosphorylation of the c-jun-N-terminal kinase (JNK) and p38 mitogen activated protein kinases (p38 MAPK) in lung endothelial cells and contributes to apoptotic response in K562 cells. Our recent studies show that in addition to causing toxicity, 4-HNE induces defense mechanisms against oxidative stress and protects the neighboring cells from apoptosis [14]. Its concentration in cells is regulated by the alpha class gluta-thione transferases, particularly GSTA4–4, that catalyze its conjugation to glutathione (GSH) with high catalytic efficiency [15–17]. In order to understand the exact role of GSTA4–4 in protection of cells against acute 4-HNE to-xicity, we isolated MEF from previously generated *Gsta*4 null mice [18]. In this study, we compared the effects of electrophile/oxidants including 4-HNE$(Ac)_3$, hydrogen peroxide (H_2O_2), and N,N'-dimethyl-4,4'-bipyridinium dichloride (paraquat) on *Gsta*4 null and WT MEF cells. 4-HNE$(Ac)_3$ is a biologically inert pro-drug form of 4-HNE that is advantageous for *in vitro* treatment of cells [5,19]. 4-HNE is highly electrophilic and may interact with cell surface proteins and components of culture medium, whereas 4-HNE$(Ac)_3$ is diffusible across the cell membrane and upon enzymatic reaction with intracellular hydrolases active 4-HNE is liberated, more closely mimicking intracellular 4-HNE formation. Sensitivity of *Gsta*4 null cells towards 4-HNE was further correlated with the accumulation of 4-HNE-protein adducts, DNA fragmentation, and activation of stress related kinases. In addition, we tested if exogenous delivery of GSTA4–4 into *Gsta*4 null cells provides protection from 4-HNE toxicity by reversing the course of stress kinase activation.

2. Materials and Methods

2.1. Materials

4-HNE$(Ac)_3$ was prepared by the method of Neely *et al*. [19]. DMEM cell culture medium, fetal bovine serum, Penicillin/streptomycin, phosphate buffered saline (PBS), proteinase K, 4% - 12% Bis-TrisNuPAGE gels, running and transfer buffers, and SYBR green were purchased from Invitrogen (Carlsbad, CA). BioTrek Protein Delivery Regent was purchased from Stratagene. Antibodies against caspase-3, JNK, ERK, and p38 MAPK were from Cell Signaling Technology (Beverly, MA) and MAPK inhibitors were purchased from EMD SERONO, INC. (Rockland, MA). Caspase-Glo® kit for the detection of caspase-3, 8, and 9 were purchased from Promega (Madison, WI). Kinase inhibitors were obtained from Calbiochem, (EMD Biochemicals, Germany). CCK-8 kit was purchased from Dojindo Molecular Technologies, Inc. (Rockville, Maryland). Polyclonal antibodies against mGSTA4–4 and hGSTA1–1 were raised in chicken and rabbit respectively and antibody against DmGSTD1–1 was a kind gift from B. J. Cochrane, University of South Florida, Tampa,FL. All other reagents and chemicals were purchased from Sigma-Aldrich (St. Louis, MO).

2.2. Mouse Embryonic Fibroblast Lines

MEF were harvested according to standard protocol [20,21]. Briefly, uteri were obtained at 13.5 days of pregnancy from wild type (WT) and *Gsta*4 null mice in 129/Sv background [18] and embryos isolated. Heads and viscera were removed and bodies were minced then digested with 0.25% trypsin/1 mM EDTA (GIBCO) for 5 – 10 min at 37°C. A single cell suspension was obtained by mixing digested bodies and complete DMEM medium.

Spontaneously immortalized cell lines were used for most experiments at passage numbers less than 20. MEFs were cultured until 80% - 85% confluent and passaged at a ratio of 1:2. Expression level of GSTA4–4 in WT and *Gsta*4 null MEF was verified by western blot using antimGSTA4–4 antibody.

2.3. Transient Transfection of *Gsta*4 Null MEF with Control and mGSTA4–4 Expression Vector

*Gsta*4 null MEF were transiently transfected with pRC/ CMV/*Gsta*4 and control plasmid constructed earlier in our lab [22] using Lipofectamine 2000 reagent (Life Technologies, Grand Island, NY). After 24 h, media containing transfection reagent and plasmid was replaced with complete DMEM and cells allowed to recover for an additional 24 h before experiments. A portion of transiently transfected cells were examined by Western blot for the expression of mGSTA4–4 before cell viability assay (data not shown).

2.4. Cell Viability Assay

Adherent MEF were trypsinized and pelleted by centri-fugation at 500 × g for 5 minutes at 2°C - 8°C and washed twice by suspending in 5 mL complete DMEM. Cell pellet was resuspendedat 2×10^5 cells/ml in DMEM and 100 μL/well were seeded in 96 well plates and allowed to recover for 16 – 18 hours before treatment. WT, *Gsta*4 null and transiently transfected *Gsta*4 null MEF cells were treated with 4-HNE $(Ac)_3$ (0 – 25 μM), and in a separate experiment WT and *Gsta*4 null MEF cells were treated with paraquat (0 – 250 μM), and H_2O_2 (0 – 700 μM) for 24 hours and analyzed for viability by MTT assay using CCK-8 kit.

2.5. Quantitation of 4-HNE-Protein Adducts by ELISA

4-HNE-protein adducts were quantitated by competitive ELISA [23] using a polyclonal antibody against 4-HNE-modified keyhole limpet hemocyanin, generously provided by Dr. Dennis R. Petersen, University of Colorado, Denver. A debris free cell lysate from control and treated MEF or known amounts of 4-HNE-casein were preadsor-bed with anti 4-HNE antibody and added to a 96 well immunoassay microplate precoated with 4-HNE modified casein (33 μg/ml per well). After incubation for 1hr, plate was washed with PBS (containing 0.05% Tween 20 and 0.25 mg/ml casein), HRP conjugated secondary antibody added to each well and incubated for an additional 30 min at RT. The plate was washed and color developed by adding 100 μl/well of TMB One Solution (Promega, Madison, WI). After blue color development (usually 4 – 5 min), the reaction was stopped by adding 1N HCl and plate read at 450 nm. Samples were quantified using the calibration curve constructed with known amounts of competitor (4HNE-casein).

2.6. Determination of Caspase Activity

Activities of caspase-3, 8, and 9 in WT and *Gsta*4 null MEF treated with/without 4-HNE$(Ac)_3$ were analyzed using Caspase-Glo® kit according to manufacturer's instructions. Briefly, 2×10^4 null or WT cells were grown for 24 h in white-walled 96 well plates compatible with luminometer (Molecular Devices, Sunnyvale, CA). After 24 h cells were treated with 20 μM 4-HNE$(Ac)_3$ for 2 h with control cells receiving an equivalent amount of DMSO. Cleavage of caspase-3 was also confirmed by Western blot analyses. For Western blot, 2×10^5 WT or *Gsta*4 null cells were plated in 100 mm dishes and after 24 h of incubation cells were treated with DMSO or 20 μM 4-HNE$(Ac)_3$ for different time points (0 – 5 hr). Following treatment cells were lysed, clarified by centrifugation, and 25 μg protein/ well separated on 4% - 12% NuPAGE gel. Anti-caspase-3 antibody was used to detect pro and cleaved caspase-3.

2.7. DNA Damage Determined by Comet Assay

DNA damage was assessed in 4-HNE(Ac)$_3$ treated/control WT and *Gsta*4 null MEF by "comet" assay (also called single cell gel electrophoresis; SCGE) according to methods described by Singh *et al*. [24] with slight modifications. In brief, control and treated cells were suspended in liquid 0.5% low melting point agarose and spread on a glass microscope slide coated with 1% (w/v) agarose. Cells were lysed (1% Triton X-100 and 1% sodium lauryl sarcosinate for 1 h at 4 degree C in dark) and DNA allowed to unwind under alkaline conditions by covering the slides with a 300 mM NaOH, 1 mM EDTA (pH > 13.0) solution for 1 hr. Following unwinding, slides underwent electro-phoresis (25 volts (∼0.74 V/cm) for 20 min), washed and stained with SYBR green for 5 min. Cells were scored for DNA damage [25] by computerized image analysis using CometScore™ Freeware (TriTek Corporation, Sumerduck, VA; http://autocomet.com).

2.8. Activation Analysis of Stress Kinases by Western Blot

For the Western blot analyses, 2×10^5 cells were plated in 100 mm dishes and after 24 h of incubation treated with inhibitors of JNK (SP600125, 50 μM), ERK (UO126, 20 μM), and p38 MAPK (SB202190, 2 μM) for 1h before treatment with 20 μM 4-HNE(Ac)$_3$. After 2 h of treatment, control and treated cells were harvested, extracts prepared in RIPA buffer (Sigma-Aldrich, St. Louis, MO), and protein content determined by the Bradford method [26]. 25 μg of cell extracts were separated by SDS-PAGE in precast NuPage 4% - 12% Bis-Tris gels (Invitrogen, Carlsbad, CA), and the electroblot probed with polyclonal antibodies against phosphorylated JNK, ERK, and p38 MAPK. A peroxidase-coupled secondary antibody and SuperSignal West Pico (Thermo scientific, Rockford, IL) with chemiluminescent detection were used for visualization of bands on a Bio-Rad imaging system (Bio-Rad Laboratories, Hercules, CA).

2.9. Effects of Kinase Inhibitors on MEF Viability

To compare the effects of JNK, ERK and p38 MAPK inhibitors on the viability of WT and *Gsta*4 null MEF, 2×10^4 cells/well were plated in 96 well plates in complete growth medium. After 24 h the cells were separately treated with inhibitors of JNK (SP600125; 50 μM), ERK (UO126; 20 μM), and p38 (SB202190; 2 μM) for 1 h and then exposed to 20 μM 4-HNE(Ac)$_3$. The viability of cells after 24 h was determined by MTT assay as described above.

2.10. Delivery of Purified GSTs into Gsta4 Null MEF Cells

The BioTrek Protein Delivery Regent (Stratagene) was used to deliver purified recombinant mouse GSTA4–4, Drosophila DmGSTD1–1 [27], and human GSTA1–1 expressed in *E. coli*. Purified GSTs were diluted to 1 mg/mL with PBS and 100 μl of the diluted protein solution transferred to the tube containing lyophilized BioTrek reagent, mixed thoroughly, and incubated at RT for 5 min. Serum-free medium was then added to a final volume of 500 μl. *Gsta*4 null MEF cells approximately 50% - 60% confluent were washed once with serum-free medium, 500 μl of fresh serum-free medium added to each well, and 500 μl of the BioTrek-protein mixture was added drop wise. After 2 h complete medium was added and cells incubated at 37°C and 5% CO_2 in a humidified incubator for 16 h before treatment with 20 μM 4-HNE(Ac)$_3$. Control WT and *Gsta*4 null MEF cells received reagent and/or treatment to match protein delivered *Gsta*4 null MEF cells. Cells were harvested 2 h after 4-HNE(Ac)$_3$ treatment for Western blot analyses.

3. Results

3.1. Expression of GSTA4–4 in WT and *Gsta*4 Null MEF Cells

Expression of GSTA4–4 isozyme in WT and *Gsta*4 null MEF cells was analyzed by Western blot using specific antibody. The immunoblot presented in Figure 1(a) showed robust expression of mGSTA4–4 in WT MEF, whereas detectable expression of this isozyme was not observed in *Gsta*4 null MEF.

3.2. *Gsta*4 Null MEF Are More Sensitive to 4-HNE and Oxidant Toxicity

In mammals, GSTA4–4 catalyzes conjugation addition of reduced glutathione to 4-HNE, the majorendproduct of peroxidative degradation of lipids and a commonly used biomarker for oxidative damage in tissue [28]. Deletion of *Gsta4* was predicted to potentiate sensitivity towards 4-HNE, and we also examined sensitivity of *Gsta4* null MEF towards other oxidants, H_2O_2 and paraquat (generates superoxide $^{\bullet}O_2^-$) known to be metabolized by catalase and superoxide dismutases (SOD). Cytotoxicity of 4-HNE(Ac)$_3$ (0 – 25 μM), H_2O_2 (0 – 700 μM), and paraquat (0 – 250 μM) were compared in WT and *Gsta4* null MEF cells by MTT assay, and IC_50 values for each treatment were calculated from the dose response curves (Table 1). Cell viability curves (Figure 1(b)) indicated that *Gsta4* null MEF cells were more sensitive than corresponding WT cells to electrophilic stress elicited by 4-HNE(Ac)$_3$, a precursor which is converted intracellularly to 4-HNE [5, 19]. The functional role of mGSAT4–4 is further confirmed by the experiment in which we compared cytotoxic effects of 4-HNE(Ac)$_3$ (0 – 25 μM) in control and m-GSTA4–4 over-expressing *Gsta4* null MEF. Results clearly indicate that over expression of mGSTA4–4 rescues *Gsta4* null MEF and protect cells from 4-HNE toxicity (Figure 1(b)). Reduced viability was also observed in *Gsta4* null MEF after exposure of cells to paraquat and H_2O_2, both oxidants which initiate lipid peroxidation (Figure 1(c), (d)).

3.3. *Gsta*4 Null MEF Cells Accumulate High Levels of 4-HNE-Protein Adducts

We have previously demonstrated that the tissues analyzed from *Gsta4* null mice have high levels of 4-HNE [18,29], known to interact with lysine, histidine, and cysteine residues of proteins and peptides [30]. To determine the status of 4-HNE-protein adducts in WT and *Gsta4* null MEF cells, we performed competitive ELISA using antibodies against 4-HNE-protein adducts. Results of these analyses (Figure 2) revealed the concentration of 4-HNE-protein adducts was significantly higher in *Gsta4* null cells. These results are consistent with the high levels of 4-HNE adducts we observed in liver, skeletal muscle and white adipose tissue of *Gsta4* null mice in 129/sv background [29].

3.4. *Gsta*4 Null MEF Are More Sensitive to 4-HNE Induced Apoptosis

Previous studies have shown that 4-HNE initiates apoptotic cell death in a wide variety of cells via the Fas-dependent extrinsic and mitochondria-mediated intrinsic pathways [14,31,32]. While in Fas-dependent extrinsic apoptosis the initiator caspase-8 plays an important role, caspase-9 has been shown to be involved in the mitochondria mediated apoptosis, however, both initiator cas-pases can lead to the activation of the executioner or effector caspases 3 and 7 [33]. Cytochrome C released from mitochondria facilitates the cleavage of pro-caspase-3 (37 kDa) to smaller fragments of 20, 17, and 12 kDa [34]. Activation of caspase-3 analyzed by fluorescence assay indicated that in *Gsta*4-null MEF cells, 4-HNE and H_2O_2 mediated induction of caspase-3 was almost 2 fold higher than WT MEF cells, indicating enhanced sensitivity to 4-HNE-induced apoptosis (Figure 3(a)). This observation was further confirmed by Western blot analysis (Figure 3(b)), which showed a time dependent increase in the cleavage of caspase-3 in null MEF cells after 4-HNE(Ac)$_3$

treatment not detected in MEF WT cells. Likewise, the activation of caspase-8 and 9 was significantly increased in MEF null cells treated with 4-HNE (Figure 3(c), (d)).

3.5. 4-HNE Causes DNA Damage

Caspases have been identified as key pro-apoptotic proteins that can disrupt essential homeostatic processes and initiate an orderly disassembly of cells, including degradation of genomic DNA. Significant activation of cas-pases in *Gsta*4 null cells by 4-HNE$(Ac)_3$ was further correlated with strand breaks in DNA. The extent of DNA damage in treated and control MEF cells was analyzed by the alkaline comet assay [35]. As shown in Figure 4, MEFs treated with vehicle alone did not show any significant DNA damage. Substantial DNA fragmentation was observed in both WT and *Gsta*4 null MEFs upon exposure to 20 μM 4-HNE$(Ac)_3$ for 2 h, however, this damage was significantly more pronounced in *Gsta*4 null cells.

3.6. Activation and Phosphorylation of ERK, JNK, and p38 MAPK Is Elevated in *Gsta*4 Null MEF

Treatment of hepatocytes or endothelial cells with 4-HNE results in phosphorylation of ERK, JNK, and p38 MAPKs [12,36], furthermore, pretreatment of bovine lung microvascular endothelial cells with inhibitors of MEK1/2, JNK, or p38 MAPK only partially attenuated 4-HNE-mediated barrier function and cytoskeletal remodeling [12]. Western blot analysis (Figure 5) indicated that even though 4-HNE treatment resulted in the activation and phosphorylation of ERK, JNK, and p38 MAPK in both WT and null MEF, the activation of these kinases was only moderately more pronounced in null cells, which could be attributed to elevated basal levels of 4-HNE. Results showed that 4-HNE induced activation of these protein kinases correlated with the increased sensitivity of null MEF to oxidant induced apoptosis. To further corre- late 4-HNE mediated induction of MAP kinases and increased cytotoxicity in *Gsta*4 null MEF, we pre-treated cells with selective and specific inhibitors of ERK (UO126) [37], JNK (SP600125) [38], and p38 MAPK (SB 202190) [39]. Pretreatment of MEF with kinase inhibitors blocked the phosphorylation of stress related kinases in cells treated with 4-HNE$(Ac)_3$ (Figure 5) and partially abrogate the cytotoxic effect in *Gsta*4 null MEF (Figure 6). Results clearly suggests that MAP kinases play an important role in 4-HNE mediated toxicity and cell death in MEF and absence of GSTA4–4 potentiates the cytotoxic effects of 4-HNE.

3.7. Delivery of GSTA4–4 into MEF Null Cells Provides Protection from 4-HNE Induced Apoptosis

In order to ascertain the protective role of GSTA4–4 against oxidant toxicity, we delivered mGSTA4–4, *Droso-phila* DmGSTD1–1, and hGSTA1–1 (catalytic efficiency towards 4-HNE diminishes respectively) into *Gsta*4 null MEF using Stratagene Bio Trek delivery system [40]. After verifying successful delivery of GSTs by Western blot (Figure 7(a)), we compared the effect of 4-HNE on the viability of GST isozyme delivered null MEF cells. Results of these studies indicated that while-cells delivered with mGSTA4–4 isozyme showed significant resistance to 4-HNE$(Ac)_3$, DmGSTD1–1 and hGSTA1–1 delivered cells did not show significant alteration in viability upon treatment with 4-HNE$(Ac)_3$ (data not shown). Effects of 4-HNE on the activation of JNK and p38 MAPK were also compared in GST isozyme delivered null MEF cells. Western blot analyses (Figure 7(b)) revealed that delivery of mammalian and invertebrate GSTs into *Gsta*4 null MEF cells indeed resulted in the attenuation of 4-HNE mediated activation of JNK, ERK1/2, and p38 MAPK in a manner dependent on the catalytic efficiency of each towards 4-HNE. Murine GSTA4–4, which has the highest catalytic efficiency towards 4-HNE (1500 S-1. mM-1 [41]), shows the highest reversal of stress kinase activation; followed by DmGSTD1–1 (399 S-1. mM-1 [27]) and

hGSTA1–1 (58.8 S-1. mM-1 [42]). Reversal by 4-HNE-metabolizing GSTs indicates that the increased activation of stress-related kinases in *Gsta*4 null MEFs is most likely due to 4-HNE and the activation of these protein kinases contributes to 4-HNE-induced apoptotic signaling.

4. Discussion

It is widely recognized that GSTs play a major role in the regulation of intracellular 4-HNE levels and in defense mechanisms against oxidative stress. The alpha class GST isozymes GSTA1–1, GSTA2–2, and GSTA3–3 efficiently catalyze the GSH dependent reduction of fatty acid hy-droperoxides [28,43] and limit the formation of 4-HNE, while the isozyme GSTA4–4 exhibits high catalytic efficiency for conjugating 4-HNE to GSH for its metabolism and disposition [41]. Thus, the alpha class GSTs limit the cellular accumulation of 4-HNE and related unsaturated lipid aldehydes associated with oxidative damage to cells via GSH peroxidase and GSH conjugating activities. The *Gsta*4 null mouse model generated by us [18] has provided significant insight into the physiological and pathological roles of 4-HNE. The observed hypersensitivity of *Gsta*4 null MEF cells to oxidants and the correlation of this sensitivity with increased accumulation of 4-HNE-protein adducts is consistent with previous findings [44, 45], suggesting that oxidative damage to cells leads to significant increases in 4-HNE adducts and overexpres-sion or induction of GSTA4–4 prevents such accretion [13]. Rapid release of reactive oxygen species such as superoxide radical and hydrogen peroxide during oxidative stress leads to formation of the lipid peroxidation product 4-HNE, which could be responsible for the impairment of downfield protective mechanisms in the cellular system [46]. Increased sensitivity of *Gsta*4 null MEF towards unrelated substrates (H_2O_2 and paraquat) of GSTA4–4 during *in vitro* treatment is possibly due to excessive production of 4-HNE by treated cells. The radical-initiated reaction with polyunsaturated fatty acids is unique since it results in a chain reaction [47], thus, 4-HNE formation will be accelerated despite the protective effects of catalase and superoxide dismutase in *Gsta*4 null cells. For this reason, cytotoxic effects of other oxidants are not surprising. These results clearly indicate that GS-TA4–4 is an important enzyme and it plays a pivotal protective role during chronic and acute oxidative stress.

Apoptosis is characterized by cell shrinkage, chromatin condensation, and blebbing of cellular components producing apoptotic bodies [48–50]. The process can be summarized as initiation by an apoptosis inducing agent, cleavage of pro-caspases constituting a family of aspartate-specific cysteine proteases resulting in a caspase cascade, and culminating with the cleavage of proteins by executioner caspases and cell death [51,52]. Supraphysio-logical concentrations of 4-HNE are known to induce apoptosis in most cell types studied to date and is induced via the death receptor Fas-mediated extrinsic and the mitochondria mediated intrinsic apoptotic pathway [52,53]. Increased apoptosis accompanied by increased 4-HNE-protein adducts and DNA damage in *Gsta*4 null MEF cells supports the pro-apoptotic role of 4-HNE. Furthermore, an accelerated activation of caspase-3, caspase-8, and cas-pase-9 is consistent with our previous findings [14,31] suggesting that 4-HNE induces apoptosis *via* both the extrinsic and intrinsic pathways in a cell type independent manner. 4-HNE$(Ac)_3$ activated caspase-3 in *Gsta*4 null cells possibly targets key enzymes responsible for DNA repair and fragmentation to execute apoptosis [51]. The observed increase in DNA fragmentation assessed by comet assay in treated *Gsta*4 null cells positively correlates with the executioner role of caspase-3.

ERK, JNK, and p38 MAPK are vital and central elements of the MAPK family, mediating multiple signaling cascades initiated by stress, cytokines, and growth factors. ERK is the final enzyme of the MAPK pathway which transmits signals into the nucleus and chronic activation of ERK can induce apoptosis [54]. Consistent with earlier studies [12,31,45],

treatment with 4-HNE resulted in the activation and phosphorylation of JNK, ERK1/2, and p38 MAPK in MEF cells. Higher basal levels of activated/ phosphorylated JNK, ERK1/2, and p38 MAPK observed in *Gsta*4 null MEFs accompanied by increased 4-HNE levels further correlates the role of 4-HNE in the activation of these kinases. Even though stress kinase activation is known to play an important role in the mechanisms of apoptosis, cell survival/proliferation, and inflammation, their ultimate effect on cellular fate is still controversial. For example, activation of different isoforms of JNK under various stimuli has been shown to affect both apoptotic and pro-survival signaling [32,55]. Our results showing that pretreatment of MEF with inhibitors of MAPK and JNK only partially abrogated the sensitivity of MEF null cells to 4-HNE$(Ac)_3$ (Figure 6) suggest 4-HNE also exerts its toxicity through undefined mechanisms in addition to MAPK and JNK mediated apoptosis.

Functional differences between WT and cells lacking GSTA4–4 could be triggered by differing levels of 4-HNE, by a change in the concentration of mGSTA4–4 substrates other than 4-HNE, or by non-catalytic functions of mGS-TA4–4, such as direct binding to proteins. GSTs of the Pi and Mu classes are known to act as stress sensors by sequestering signaling kinases under normal conditions and releasing them in response to stress [56–62]. GSTs may also serve an anti-apoptotic role, one example is a novel plant GST shown to suppress Bax, and thus affect apop-tosis [63–65]. To distinguish between the various modes of GST action, we directly introduce mGSTA4–4 (which has 4-HNE-conjugating ability), the phylogenetically distant *Drosophila* DmGSTD1–1 (which is also capable of conjugating 4-HNE, albeit with a lesser catalytic efficiency than mGSTA4–4) [27], and hGSTA1–1 (which is closely related to mGSTA4–4 but lacks significant activity toward 4-HNE) into *Gsta*4 null cells. mGSTA4–4 and, to a lesser extent, consistent with its lower activity, DmGSTD1–1 were able to abrogate the activation of p38 MAPK and JNK (Figure 7) by 4-HNE$(Ac)_3$, whereas hGSTA1–1 had no effect. *Drosophila* DmGSTD1–1 belongs to a different family of GSTs than mGSTA4–4 [27], and is unlikely to enter into specific protein-protein interactions in a mammalian system. Thus, the only known property of the three enzymes that correlates with the ability to prevent stress kinase activation is conjugation of 4-HNE, indicating that 4-HNE mediates kinase activation. Together, results of present studies clearly demonstrate that 4-HNE significantly contributes to the cytotoxicity and that GSTA4–4 plays a crucial role in nullifying acute 4-HNE toxicity by converting it to non-electrophilic glu-tathione conjugate glutathionyl-HNE (GS-HNE).

5. Concluding Remarks

GSTA4–4 is a key enzyme that regulates 4-HNE concentration in mammalian cellular systems. Our findings clearly suggest that the impairment of 4-HNE conjugation in *Gsta*4 null MEF increases the sensitivity towards 4-HNE by activating caspases and stress-activated kinases. Furthermore, the positive relationships between DNA damage, increased 4-HNE-protein adduct accumulation, and apoptosis in *Gsta*4 null MEF upon treatment with 4-HNE$(Ac)_3$ were demonstrated. These results suggest that 4-HNE induced apoptosis of *Gsta*4 null MEF is associated with the enhanced accumulation of 4-HNE-protein ad-ducts, DNA damage, and the activation of caspases-3, 8 and 9. We also demonstrated that exogenous delivery of GST isozymes with medium to high catalytic efficiency towards 4-HNE prevents stress-related kinase activation upon treatment with HNE$(Ac)_3$. Thus, we conclude that GSTA4–4 is a major 4-HNE metabolizing enzyme in the mouse, which protects cells from 4-HNE toxicity during acute oxidative stress.

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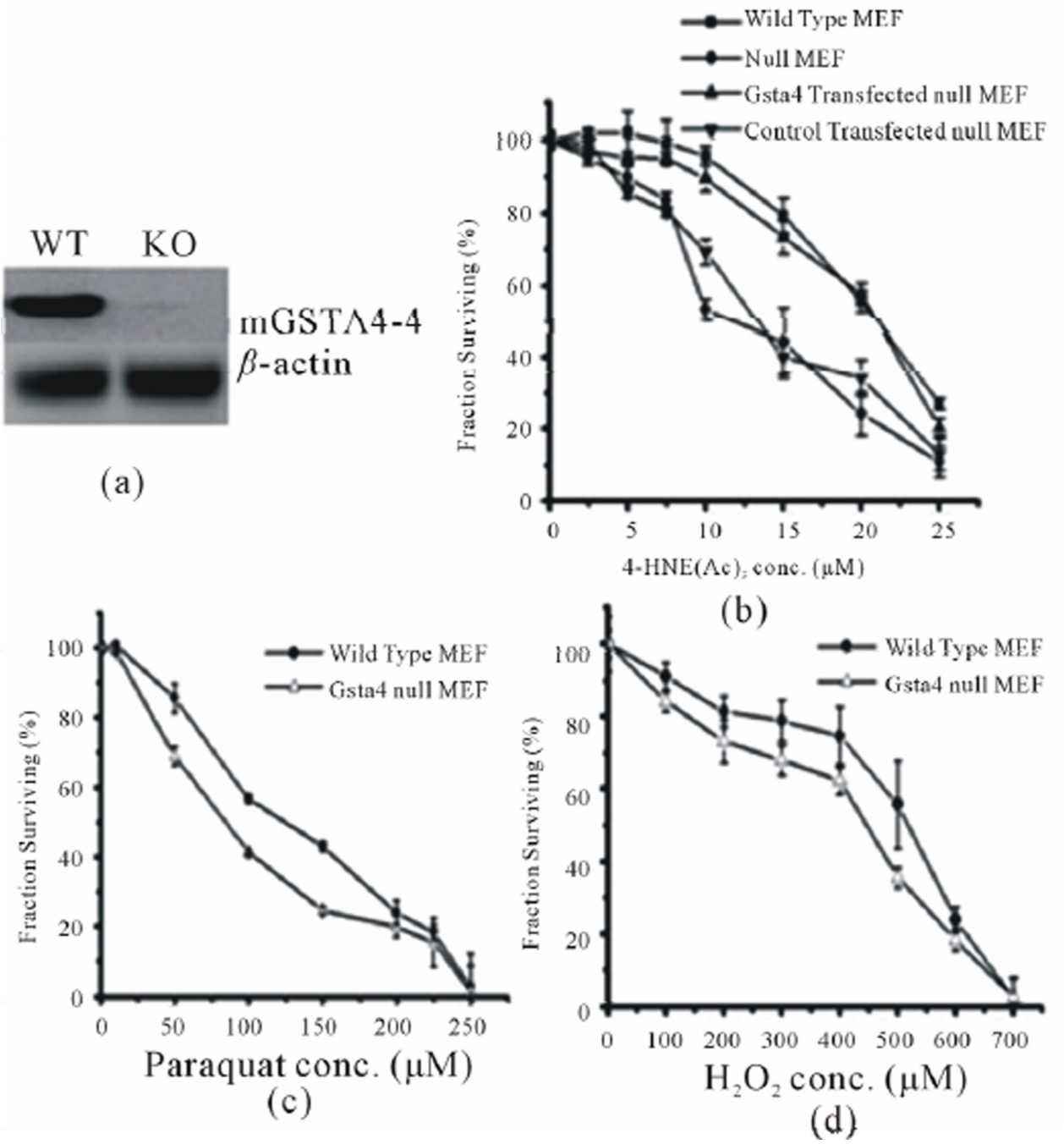

Figure 1.

(a): Expression of GSTA4–4 in MEF cells prepared from WT and *Gsta*4 null mice analyzed by Western blot; (b)-(d): Viability of wild-type and *Gsta*4 null MEF cells analyzed with the CCK-8 kit after treatment with different concentrations of 4-HNE$(Ac)_3$ (0 – 25 μM) , paraquat (0 – 250 μM) and H_2O_2 (0 – 700 μM).

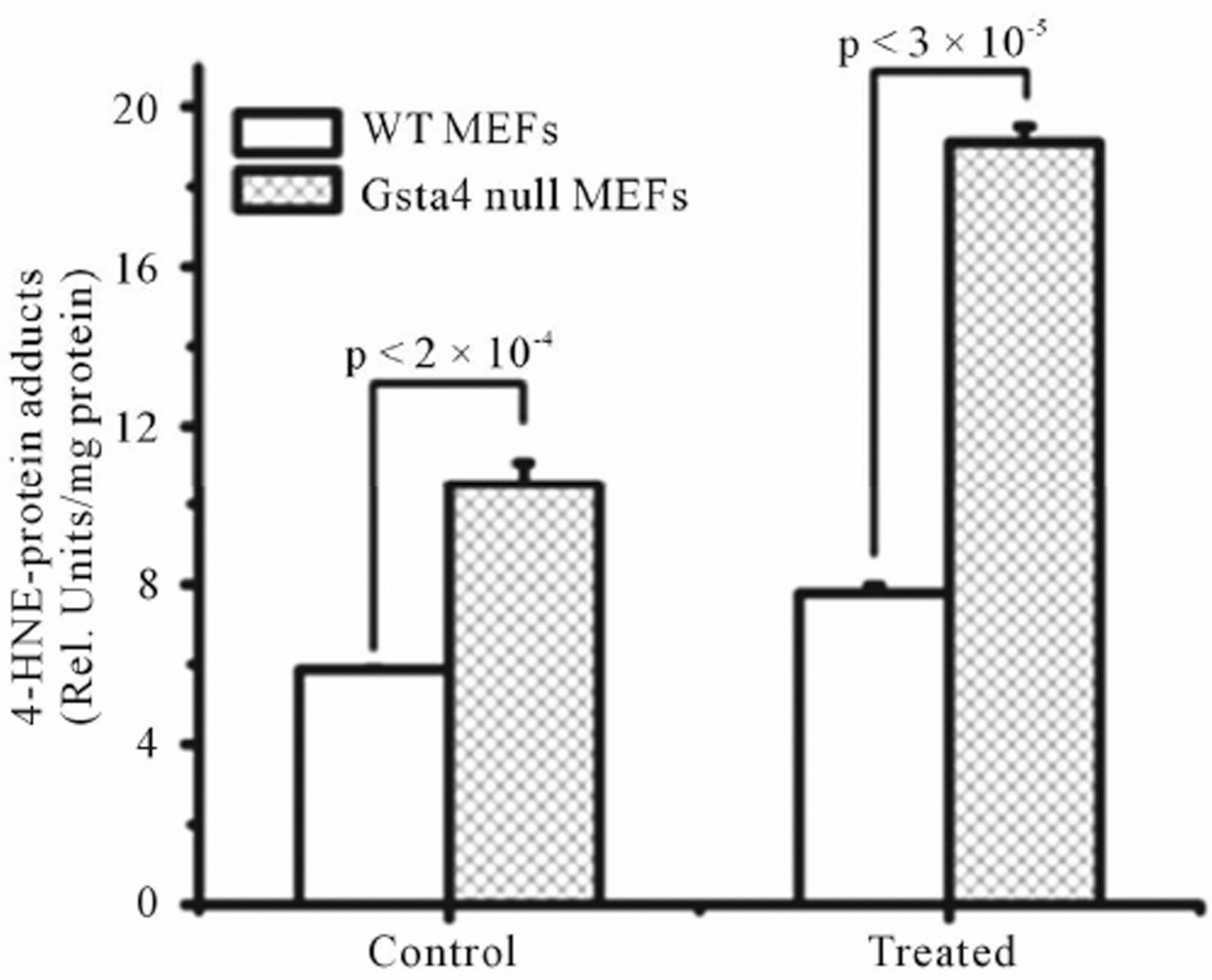

Figure 2.

Analysis of 4-HNE-protein adducts in control and treated MEF cells by ELISA: Cells (2×10^6) were incubated with 20 μM 4-HNE(Ac)$_3$ for 2 h and subjected to ELISA analysis using antibodies against 4-HNE protein adducts as described in Methods section. Data presented are Mean ± SD of two separate experiments done in triplicate (n = 6).

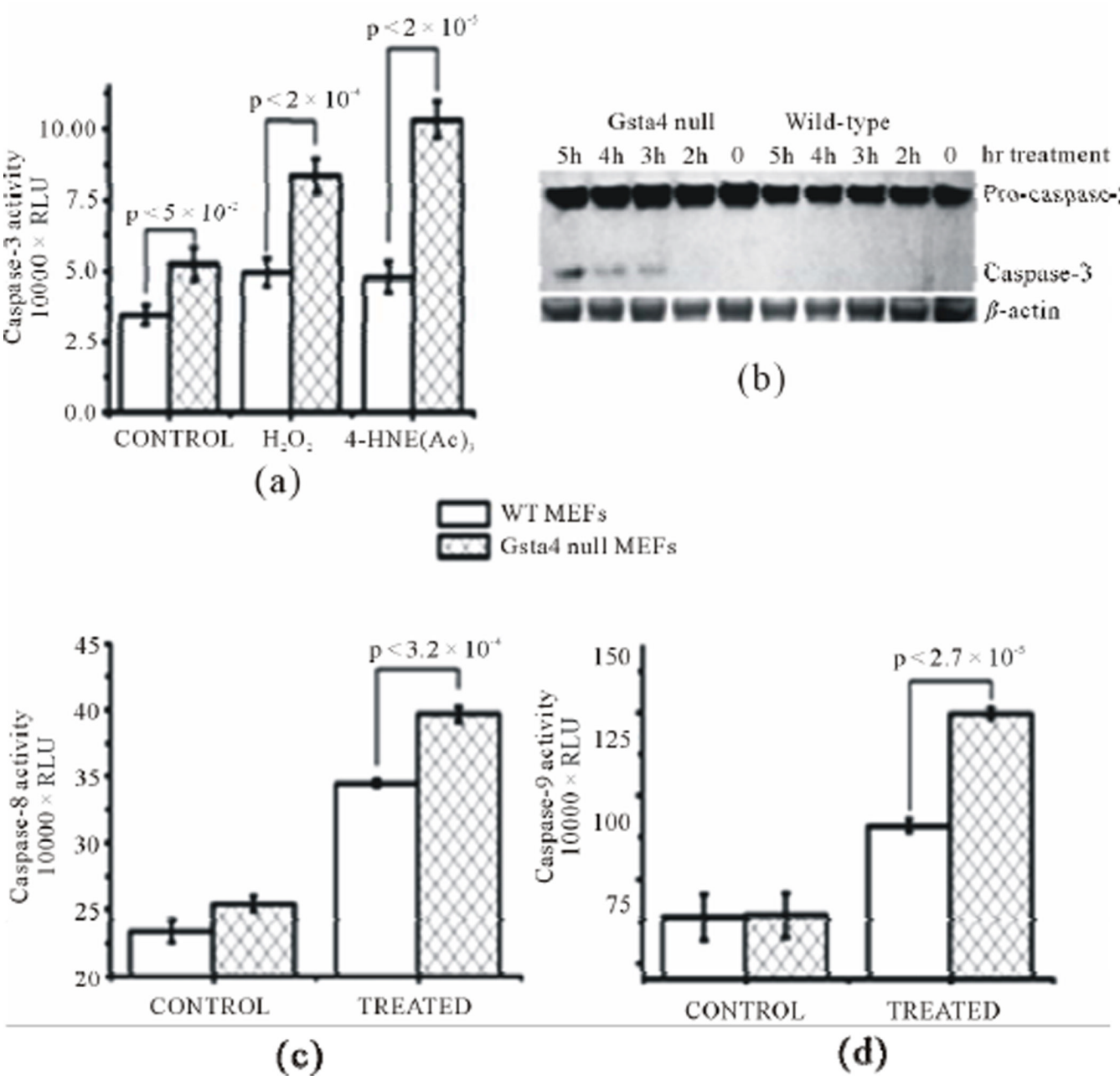

Figure 3.

4-HNE or H_2O_2 induced activation of caspases in MEF cells; (a) Activation of caspase-3 in MEF cells treated with H_2O_2 (100 μM) and 4-HNE$(Ac)_3$ (20 μM) measured by ELISA; (b) Western blot analyses of 4-HNE induced activation of caspase3. Cells were treated with 4-HNE$(Ac)_3$ (20 μM) for 0 h - 5 hr, harvested, washed, and lysed in RIPA buffer. 25 μg of protein from control and treated samples were resolved by SDS-PAGE and immunobloted on nitrocellulose membrane. Immunoblots were probed with caspase3 antibodies; (c) and (d). 4-HNE induced activation of cas-pase-8 and 9 in MEF cells: WT and null MEF cells (5000 cells) were treated with 4-HNE$(Ac)_3$ (20 μM) for 2 h. Control and 4-HNE treated cells were analyzed for the activation of caspase-8 and 9 by ELISA as described in the

Methods section. Data presented are Mean ± SD of two separate experiments done in quadruplicates (n = 8).

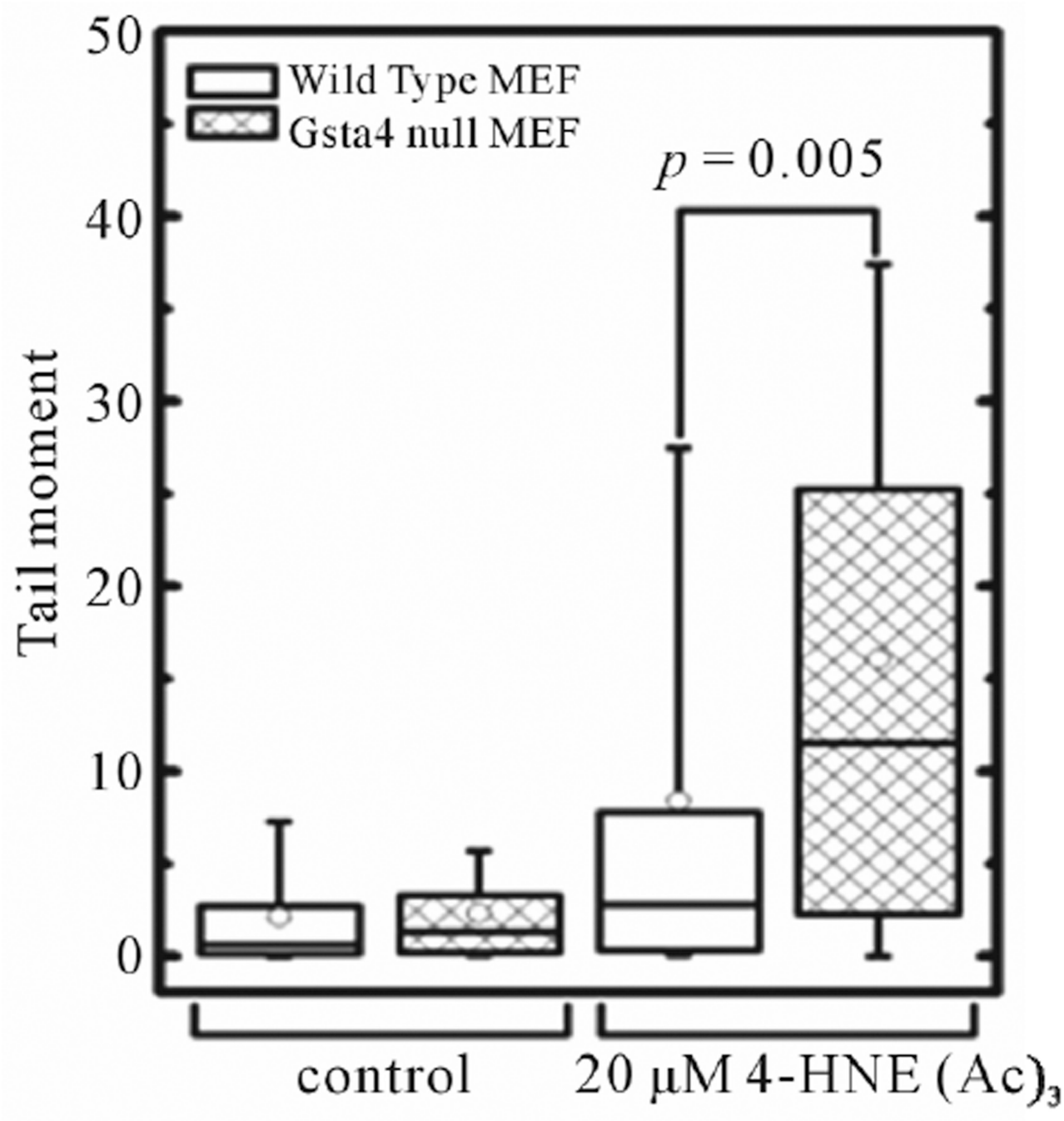

Figure 4.

DNA damage determined by alkaline comet assay. MEFs from wild-type and *mGsta*4 null mice were treated for 4 hr with vehicle or with 4-HNE(Ac)$_3$. 60 cells from each treatment were scored for DNA damage [25] using Comet-Score (http://autocomet.com). Box: 25 – 75 percentile; whiskers: 10 – 90 percentile; horizontal line: median, circle: mean. Untreated WT and *mGsta4*null cells do not differ ($p > 0.4$), but cells treated with 20 μM 4-HNE(Ac)$_3$ are significantly different ($p = 0.005$, Wilcoxon rank-sum test).

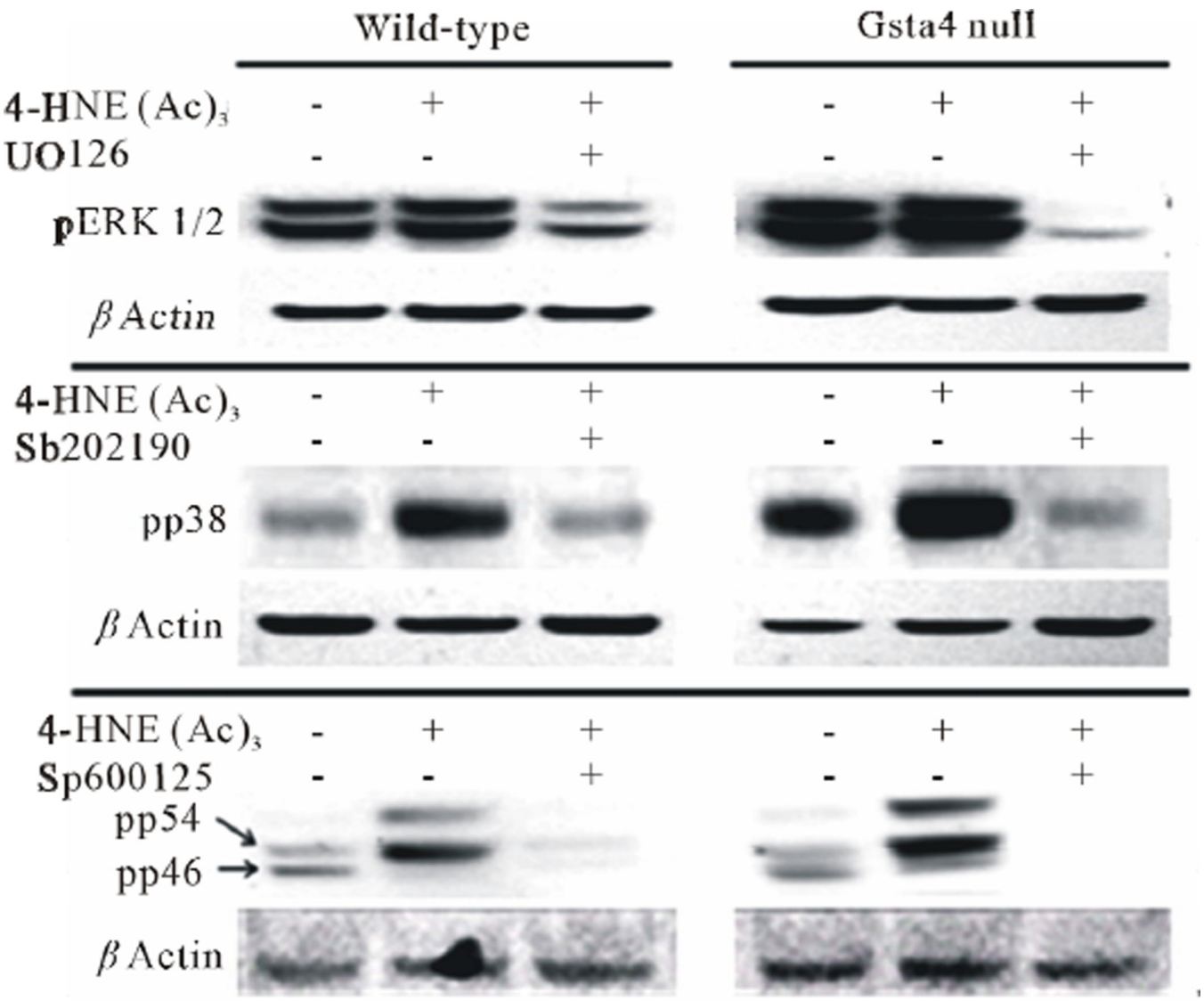

Figure 5.

Activation of MAP kinases in MEF cells after treatment with kinase inhibitors and 4-HNE(Ac)$_3$. Cells (2×10^6) were treated separately with the SP600125 (JNK inhibitor), UO126 (ERK inhibitor) and SB202190 (p38 inhibitor) and exposed to 4-HNE(Ac)$_3$ (20 μM) for 2 hr. Cells treated only with 4-HNE(Ac)$_3$ and vehicle alone were used as controls. Extracts of control and treated cells were subjected to Western blot analyses and probed separately with antibodies against pERK [anti-pp44/42(Thr202/Tyr204)], p38 [anti-phospho-Thr180/Tyr204] and pJNK [anti-pJNK(Thr183/ Tyr185)]. Antibodies against *β* actin were used to ascertain equal loading of proteins. Appropriate lanes in the Western blot have been marked.

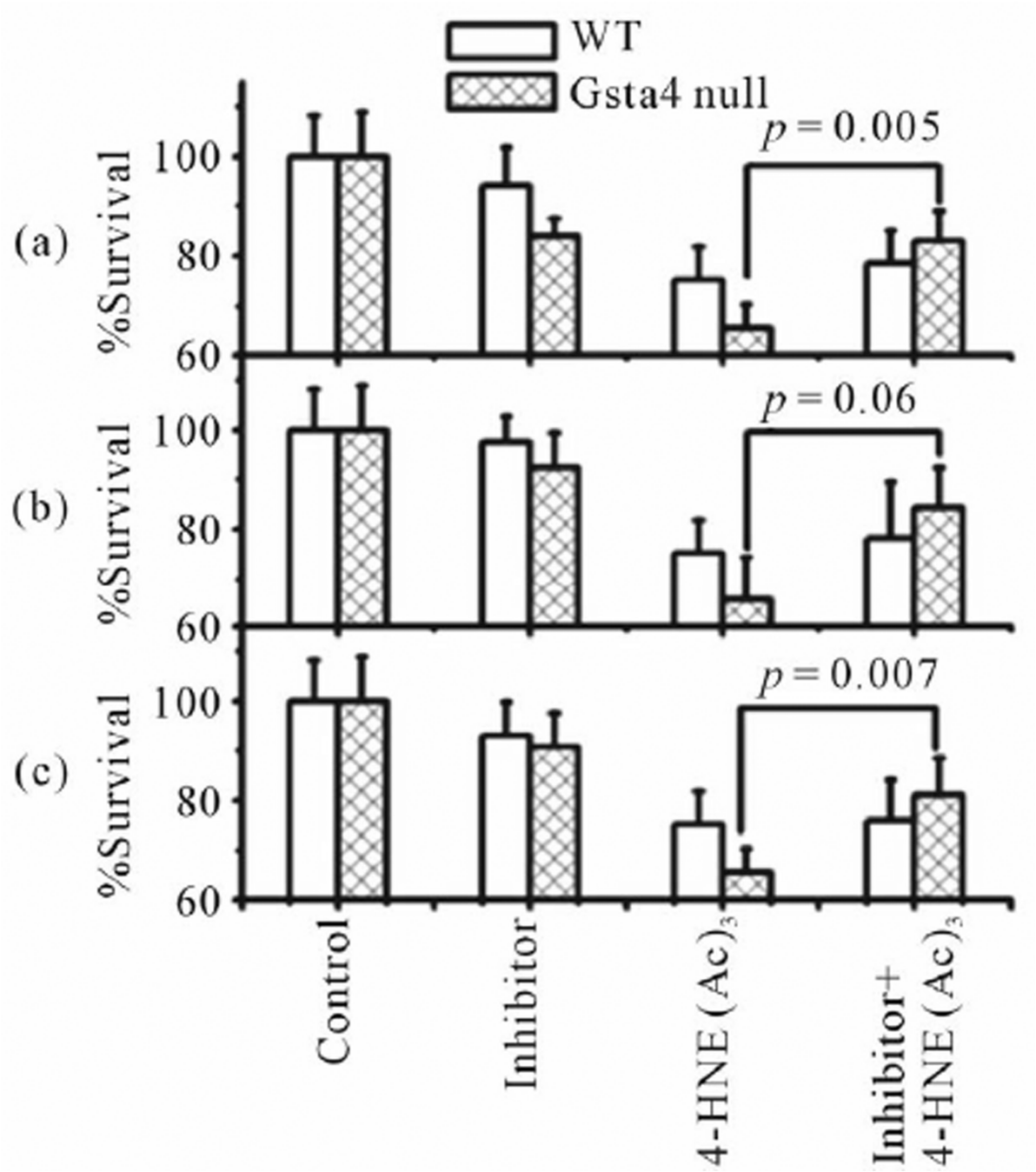

Figure 6.

Effect of inhibitors of p38 (a); ERK (b) and JNK (c) on viability of MEF cells:WT and null cells (2×10^4) were seeded in a 96 well plate and treated with fixed concentration of inhibitors for 1 h then treated with 10 μM of 4-HNE$(Ac)_3$ for 24 h. Control cells received equal volume of DMSO. After completion of treatment cell viability was assayed by MTT as described in the Methods section. Data shown are Mean ± SD of two experiments with eight replicate wells.

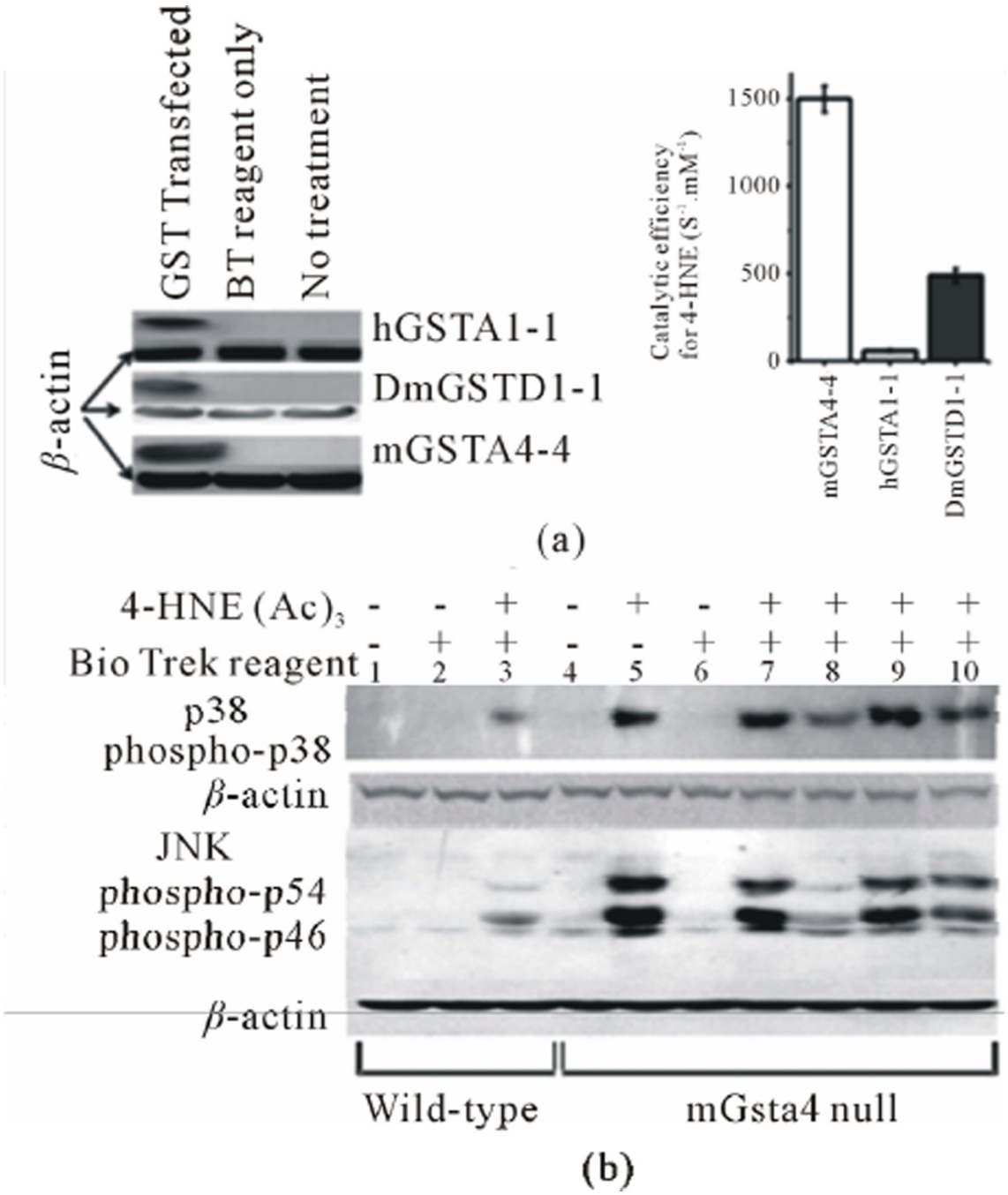

Figure 7.

(a) Verification of GST isozyme uptake in *Gsta*4 null MEF cells after delivery with BioTrek reagent: *mGsta*4–4, hGSTA1–1, and DmGSTD1–1 were delivered into *Gsta*4 null MEF cells using BioTrek (BT) reagent as described in Methods section. Delivery of each isozyme in *Gsta*4 null MEF was confirmed by Western blot analyses of GST tran-sfected and control cell extracts (BT reagent treated and untreated). (b) Effect on stress kinase activation by exogenous introduction of GST protein into *Gsta*4 null MEF cells: Lane 1: untreated wild-type cells. Lane 2: wild-type cells treated with protein delivery reagent only. Lane 3: wild-type cells treated with protein delivery reagent and 20 μM 4-HNE(Ac)$_3$. Lanes 4–10:*mGsta*4

null cells. Lane 4: untreated. Lane 5: treated with 20 μM 4-HNE$(Ac)_3$. Lane 6: treated with delivery reagent only. Lane 7: treated with delivery reagent and 20 μM 4-HNE$(Ac)_3$. Lanes 8–10: treated with 20 μM 4-HNE$(Ac)_3$ with delivery reagent plus 100 μg/dish of mGSTA4–4 (lane 8), hGSTA1–1 (lane 9), or DmGSTD1–1 (lane 10), respectively. The core comparison: delivery of no protein (lane 7) or the three GSTs into KO cells (lane 8–10) exposed to 4-HNE$(Ac)_3$.

Table 1

IC_{50} values of 4-HNE$(Ac)_3$, H_2O_2, and Paraquat analyzed for MEF cells isolated from wild type and *Gsta*4 null mice. Data presented are Mean ± SD of three separate experiments done in quadruplicates (n = 12).

Treatment	IC_{50} (μM) ± SD	
	Wild type	***Gsta4* null**
4-HNE$(Ac)_3$	21.6 ± 1.65	11.7 ± 1.15
H_2O_2	527 ± 32	403 ±25
Paraquat	125 ± 15	80 ± 8