Human GSTA1-1 reduces c-Jun N-terminal kinase signalling and apoptosis in Caco-2 cells

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The effect of GSTA1-1 (glutathione S-transferase Alpha 1-1) on JNK (c-Jun N-terminal kinase) activation was investigated in Caco-2 cells in which *GSTA1* expression increases with degree of confluency, and in MEF3T3 cells with Tet-Off-inducible *GSTA1* expression. Comparison of *GSTA1* expression in pre-confluent, confluent and 8-day post-confluent Caco-2 cells revealed progressively increasing mRNA and protein levels at later stages of confluency. Exposure of pre-confluent cells to stress conditions including IL-1 β (interleukin-1 β), H₂O₂ or UV irradiation resulted in marked increases in JNK activity as indicated by c-Jun phosphorylation. However, JNK activation was significantly reduced in post-confluent cells exposed to the same stresses. Western-blot analysis of GSTA1-1 protein bound to JNK protein pulled down from cellular extracts showed approx. 4-fold higher GSTA1-1–JNK complex formation in post-confluent cells compared with pre-confluent cells. However, stress conditions did not alter the amount of

GSTA1-1 bound to JNK. The role of GSTA1-1 in JNK suppression was more specifically revealed in Tet-Off-inducible MEF3T3–GSTA1-1 cells in which *GSTA1* overexpression significantly reduced phosphorylation of c-Jun following exposure to IL-1 β , H₂O₂ and UV irradiation. Finally, the incidence of tumour necrosis factor α /butyrate-induced apoptosis was significantly higher in pre-confluent Caco-2 cells expressing low levels of *GSTA1* compared with post-confluent cells. These results indicate that GSTA1 suppresses activation of JNK signalling by a pro-inflammatory cytokine and oxidative stress and suggests a protective role for GSTA1-1 in JNK-associated apoptosis.

Key words: apoptosis, c-Jun N-terminal kinase (JNK), extracellular-signal-regulated kinase (ERK), glutathione S-transferase Alpha 1-1 (GSTA1-1), interleukin-1 β (IL- β), oxidative stress.

INTRODUCTION

GSTs (glutathione S-transferases) are a large family of functionally diverse enzymes that catalyse the conjugation of glutathione with a wide variety of electrophilic compounds. As phase II enzymes, GSTs play a cytoprotective role in the detoxification of xenobiotics including toxins and carcinogens and endogenous compounds such as organic hydroperoxides produced during oxidative stress [1,2]. GSTs have recently been identified as inhibitors of stress-activated kinase activity, thereby protecting cells against apoptosis in response to cellular stress such as the formation of ROS (reactive oxygen species) [3].

MAPKs (mitogen-activated protein kinases) play a central role in transducing various extracellular signals into the nuclei, thus participating in the regulation of a variety of cellular processes [4]. In mammalian cells, four major MAPK cascades have been well characterized including ERKs (extracellular-signal-regulated kinases), p38 MAPKs, JNKs [c-Jun N-terminal kinases; also known as SAPKs (stress-activated protein kinases)] and the BMK1 (big MAPK1/ERK5) [5]. These kinase pathways are structurally related, but functionally divergent. ERKs are activated by various cytokines and growth factors, and play a central role in cell growth and differentiation. JNKs and p38 MAPKs are activated by environmental stressors such as UV irradiation, X-rays, heat and osmotic shock, oxidative stress, and pro-inflammatory cytokines such as TNF α (tumour necrosis factor α) and IL-1 β (interleukin-1 β). These cascades are involved in the

control of stress adaptation, development, immune activation, cell death and survival [6,7].

Isoenzymes from several GST classes have been shown to be JNK inhibitors. The first to be identified, GSTP1-1 (GST Pi 1-1), inhibits JNK through a mechanism involving protein–protein interactions [3]. Under conditions of oxidative stress, GSTP1-1 dissociates from JNK and forms dimers, whereas JNK recovers its functional ability to be phosphorylated by MAPKKs (MAPK kinases) and to phosphorylate c-Jun. Studies in rat and mouse cell lines have shown that increased *GSTP* expression results in decreased JNK activity and protects cells from ROS- and dopamine-mediated cell death [8–10]. Similarly, transfection of *mGsta4* into human myeloid and murine endothelial cell lines prevents 4-hydroxynonenal-induced apoptosis by inhibiting JNK-mediated signalling [11]. Moreover, overexpression of hGSTA2-2 (human GSTA2-2) protects human erythroleukaemia cells (K562) from H₂O₂-induced apoptosis by suppressing SAPK/JNK activation [12]. GSTM1-1 (GST Mu 1-1) also regulates cell signalling by inhibiting ASK1 (apoptosis signal-regulating kinase 1) [13]. Each of these studies indicates a role of GSTs as proteins implicated in the regulation of stress kinase activity; however, further investigation on classes and isoenzymes participating in this function is required.

The purpose of the present study was to investigate the role of GSTA1-1 in modulating stress-mediated activation of JNK. We hypothesized that overexpression of human GSTA1 inhibits JNK activation, reduces c-Jun phosphorylation and diminishes

Abbreviations used: ASK1, apoptosis signal-regulating kinase 1; DAPI, 4',6-diamidino-2-phenylindole; DMEM, Dulbecco's modified Eagle's medium; ERK, extracellular-signal-regulated kinase; FBS, fetal bovine serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GST, glutathione S-transferase; GSTA1-1, GST Alpha 1-1; GSTM1-1, GST Mu1-1; GSTP1-1, GST Pi 1-1; hGSTA2-2, human GSTA2-2; IL-1 β , interleukin-1 β ; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; ROS, reactive oxygen species; RT, reverse transcriptase; SAPK, stress-activated protein kinase; TNF α , tumour necrosis factor α ; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelling.

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apoptosis induced by cellular stresses including IL-1 β , UV irradiation or H₂O₂. This hypothesis was investigated in Caco-2 cells with variable GSTA1-1 expression at different stages of confluency, and in MEF3T3 cells with Tet-Off-inducible GSTA1-1 expression. Our results demonstrate the capacity of GSTA1 to bind JNK and inhibit stress-mediated activation. Furthermore, JNK-mediated apoptosis due to exposure to TNF α /butyrate is reduced in Caco-2 cells with high GSTA1-1 expression.

MATERIALS AND METHODS

Materials

The response plasmid pTRE-tight and the MEF3T3 Tet-Off cell line as well as tetracycline-free Tet system-approved FBS (fetal bovine serum) were purchased from BD Biosciences (Mississauga, ON, Canada). Human colon adenocarcinoma (Caco-2) cells were obtained from the American Type Culture Collection (Manassas, VA, U.S.A.).

RNA isolation and real-time RT (reverse transcriptase)-PCR analysis

Total RNA was isolated using TRIzol[®] reagent (Invitrogen, Burlington, ON, Canada), according to the manufacturer's instructions, and concentrations were determined by absorbance at 260 nm. Isolated RNA (1 μ g/20 μ l of reaction volume) was used for first-strand cDNA synthesis using 0.1 μ g of random primers, 20 units of RNase inhibitor and 200 units of MMLV (Moloney-murine-leukaemia virus) RT (Invitrogen). Real-time PCR was performed on a Roche Light Cycler[®] using a DNA Master SYBR Green I kit (Roche Diagnostics, Mississauga, ON, Canada). The PCR reaction was done in a volume of 10 μ l, containing 1 μ l of SYBR Green I, 5 μ M of each primer and 2 mM Mg²⁺. Oligonucleotide primers for GSTA1 were: 5'-AGCCG-GGCTGACATTCATCT-3' (sense) and 5'-TGGCCTCCATGAC-TGCGTTA-3' (antisense); and for GAPDH (glyceraldehyde-3-phosphate dehydrogenase) were 5'-ACAGTCCATGCCAT-CACTGCC-3' (sense) and 5'-GCCTGCTTACCACCTTCTTG-3' (antisense). The PCR parameters were 95 °C for 1 min, 1 cycle, and 35 cycles of 95 °C for 15 s, 70 °C for 5 s and 92 °C for 15 s. mRNA levels were normalized against GAPDH mRNA.

SDS/PAGE and Western-blot analysis

GSTA1-1 protein was identified by Western-blot analysis as described previously [14]. Briefly, 5 μ g of protein from cell extracts was separated by SDS/PAGE (12 % gel), transferred on to nitrocellulose and incubated for 1 h with a rabbit anti-(human GSTA1-1) polyclonal antibody (Oxford Biomedical Research), diluted 1:2000. After incubation with a horseradish-peroxidase-conjugated anti-rabbit secondary antibody (Vector Laboratories, Burlington, ON, Canada), bands were detected by chemiluminescence (ECL[®] Plus; GE Health Sciences, Piscataway, NJ, U.S.A.) and visualized using a Typhoon 9410 scanner (GE Health Sciences). The relative concentration of GSTA1-1 protein levels was then determined by densitometry. Three independent experiments were performed, and Western blots are presented from one representative experiment.

Cell culture and Tet-Off system

A cell line in which *GSTA1* overexpression is controlled by doxycycline was developed using the Tet-Off Gene Expression system (Clontech, Mountain View, CA, U.S.A.). Immortalized mouse embryonic fibroblast (MEF3T3) Tet-Off cells that stably express the pSV40-Hyg plasmid were grown in DMEM

(Dulbecco's modified Eagle's medium), supplemented with 10 % (v/v) Tet-Off system-approved FBS containing 50 international units/ml penicillin, 50 μ g/ml streptomycin and maintained at 37 °C and 5 % CO₂. The MEF3T3 Tet-Off cells were co-transfected with 8 μ g of pTRE/*GSTA1* plasmid and 0.5 μ g of pTK-Hyg plasmid using Lipofectamine[™] reagent (Invitrogen) to produce MEF3T3-GSTA1 cells. At 2 days after transfection, cells were grown in the presence of doxycycline hydrochloride (1 μ g/ml) and hygromycin B (200 μ g/ml) (both from Clontech). The resulting hygromycin-resistant colonies were expanded and maintained in medium supplemented with hygromycin (200 μ g/ml) and doxycycline (1 μ g/ml). To induce the expression of GSTA1-1 protein, cells were cultured in the absence of doxycycline for 48 h.

Caco-2 cells were cultured in DMEM supplemented with 10 % FBS and penicillin and streptomycin (100 μ g/ml) under 5 % CO₂ at 37 °C. The cells were plated in 100 mm dishes and were cultured until 80 % confluent (pre-confluent cells), confluent or 8 days post-confluent (post-confluent cells).

Activation of JNK by UV irradiation, H₂O₂ and IL-1 β

Medium from pre-confluent and post-confluent Caco-2 cells, and in MEF3T3-GSTA1 cells (with or without doxycycline), was replaced with PBS, and cells were exposed to UV light (60 J/m²) for 15 s with the lids to the culture dishes removed. After this, PBS was removed, fresh medium was added and cells were incubated again at 37 °C with 5 % CO₂ for 45 min before harvesting. Pre- and post-confluent cells and MEF3T3-GSTA1 cells (with or without doxycycline) were incubated in the presence of H₂O₂ (100 μ M) for 1 or 3 h before harvesting. Pre- and post-confluent Caco-2 cells were treated with IL-1 β (10 ng/ml) for 24 h.

Determination of JNK activity

JNK activity was determined using a SAPK/JNK assay kit from Cell Signaling Technology (Beverly, MA, U.S.A.). Briefly, cells were harvested under non-denaturing conditions as per the manufacturer's instructions. Cell extracts were incubated with c-Jun fusion protein beads with gentle rocking overnight at 4 °C to pull-down JNK. Kinase reactions were then performed in the presence of excess ATP for 30 min and terminated by addition of SDS sample buffer. Samples were electrophoresed on SDS/12 % polyacrylamide gels, and c-Jun phosphorylation was selectively measured by Western blotting using a phospho-Ser⁶³ c-Jun antibody and a horseradish-peroxidase-conjugated anti-rabbit secondary antibody. Blots were developed by enhanced chemiluminescence (ECL[®] Plus; GE Healthcare) and bands representing phospho-c-Jun were visualized by autoradiography and quantified by densitometry. Three independent experiments were performed and blots are presented from one representative experiment.

Phosphorylation of c-Jun was also assessed in a functional assay using the PathDetect c-Jun *trans*-Reporting System (Stratagene, La Jolla, CA, U.S.A.). MEF3T3-GSTA1 cells were plated on 24-well plates at a density of 80000 cells/well and transfected in suspension in Tet-Off system-approved FBS-DMEM medium with pFA2-cJun plasmid (fusion *trans*-activator plasmid) that encodes a fusion protein of c-Jun and the yeast GAL4 DNA-binding domain as well as the pFR-Luc (where Luc is luciferase) plasmid (reporter plasmid) that contains five tandem repeats of binding sites for GAL4 that control firefly luciferase expression. Phosphorylation of c-Jun leads to activation and binding to the GAL4 element with subsequent activation of the luciferase gene. All cells were also co-transfected with pRL-TK, a plasmid that encodes *Renilla* luciferase, to allow for normalization of transfection efficiency and cell density. Values obtained for firefly

luciferase activity were normalized to that of *Renilla* luciferase activity for each sample.

Determination of apoptosis

Cell death due to apoptosis was determined in Caco-2 cells by TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelling) and caspase 3 activation using triplicates of > 5000 cells per measurement. TUNEL was performed using an *In Situ* Cell Death Detection kit, Fluorescein (Roche Applied Science), according to a method described previously with some alterations [15]. Caco-2 cells were cultured in DMEM with 10% FBS and penicillin and streptomycin (100 µg/ml) under 5% CO₂ at 37°C in six-well plates containing sterile coverslips. Pre- and post-confluent cells were treated with TNFα (50 ng/ml) and sodium butyrate (10 mM) to stimulate apoptosis. After 24 or 48 h, medium was removed and cells were rinsed with PBS and allowed to air-dry for 1 h. Cells were then fixed with 4% (v/v) formaldehyde in PBS for 1 h at room temperature (21°C). The fixation solution was replaced with permeabilization solution (sodium citrate and Triton X-100) and incubated for 10 min on ice. Positive controls were incubated with 100 µl of DNase solution. TUNEL labelling reaction mix (50 µl) containing 5 µl of enzyme solution and 45 µl of label solution (as supplied) was added to each coverslip, except for the negative control which received only 50 µl of label solution, and incubated for 1 h at 37°C in a humidified chamber. Coverslips were then transferred to 2 ml of stop buffer (as supplied) and incubated for 5 min at room temperature. Coverslips were then washed three times with PBS before counterstaining with DAPI (4',6-diamidino-2-phenylindole) solution (1:4000 dilution in PBS) and mounted with Vectashield® on glass microscope slides.

Apoptosis was also assessed by immunofluorescent labelling of active caspase 3. Cells were cultured and fixed as described above for TUNEL staining and immunofluorescent labelling of active caspase 3 was performed using an anti-(caspase 3) antibody (AF835) from R&D Systems as described previously [15]. Apoptosis was evaluated by fluorescence microscopy (Olympus BX61), and images were acquired using the software Metamorph (Meta Imaging, series 5.0).

Statistical analysis

One-way ANOVA was used to assess statistically significant differences among treatment groups. For each statistically significant effect, the Fisher's least significant test was used for comparison between multiple group means. Significance was established at *P* values < 0.05.

RESULTS

GSTA1 expression increases progressively with stage of confluency in Caco-2 cells

We investigated the influence of *GSTA1* expression on JNK activation in Caco-2 cells as they represent a unique model of variable GST Alpha expression that is dependent on stage of confluency. Initially, it was necessary to confirm that progressive increases in *GSTA1* expression occur as Caco-2 cells differentiate and acquire an intestinal phenotype after they reach confluency. *GSTA1* mRNA and GSTA1-1 protein levels were assessed by real-time PCR and Western-blot analysis respectively in pre-confluent, confluent and post-confluent Caco-2 cells as described in the Materials and methods section. *GSTA1* mRNA levels progressively increased by 4.3-fold (*P* < 0.01) and 6.8-fold (*P* < 0.001) in confluent and post-confluent cells respectively

Figure 1 GSTA1 mRNA and GSTA1-1 protein levels increase progressively in post-confluent Caco-2 cells

(A) Total RNA was isolated from Caco-2 cells that were pre-confluent, confluent and post-confluent and *GSTA1* mRNA levels were quantified by real-time PCR. (B) GSTA1-1 protein levels in cell extracts were determined by Western-blot analysis. Band intensities were assessed by densitometry and expressed relative to untreated controls. Results are the means for three independent experiments. Significant differences from control are designated as ** (*P* < 0.01) or *** (*P* < 0.001). Significant differences from confluent cells are designated as † (*P* < 0.01).

compared with pre-confluent cells (Figure 1A). Levels of GSTA1-1 protein (Figure 1B) were also significantly elevated (*P* < 0.001) in confluent (1.7-fold) and post-confluent cells (2-fold).

JNK activation is reduced in post-confluent Caco-2 cells expressing high levels of GSTA1-1

The role of GSTA1-1 in controlling JNK activation was assessed by exposing pre-confluent and post-confluent Caco-2 cells to various diverse stress stimuli including IL-1β, UV light and H₂O₂. Treatment with IL-1β resulted in a 10-fold increase in JNK activity in pre-confluent cells (*P* < 0.001), and a 3-fold increase (*P* < 0.001) in post-confluent cells, which was significantly less (*P* < 0.001) than in pre-confluent cells (Figure 2). A 28-fold increase (*P* < 0.001) in JNK activity occurred 1 h after exposure of pre-confluent cells to H₂O₂. This was reduced to a level that was 11-fold higher (*P* < 0.1) than control cells by 3 h. The increase in JNK activity was significantly less (*P* < 0.001) in post-confluent compared with pre-confluent cells at both 1 and 3 h following exposure to H₂O₂. JNK activity was increased 20-fold (*P* < 0.001) in H₂O₂-treated post-confluent cells; however, JNK activity had returned to control levels by 3 h. Exposure to UV light increased JNK activity 45-fold (*P* < 0.001) in pre-confluent cells; however, only a 9-fold increase (*P* < 0.001) was detected in post-confluent cells. This change was significantly less (*P* < 0.001) than the increase observed in post-confluent cells.

Formation of GSTA1-1-JNK complexes is greater in post-confluent than pre-confluent Caco-2 cells and is not altered by cellular stresses

To determine whether reductions in JNK activation in post-confluent Caco-2 cells was due to increased formation of complexes between GSTA1-1 and JNK, Western-blot analysis was performed on samples containing JNK pulled down from

Figure 2 JNK activation is suppressed in post-confluent Caco-2 cells exposed to various cellular stresses

Pre-confluent (Pre) and post-confluent (Post) Caco-2 cells were exposed to (A) IL-1 β , (B) UV irradiation or (C) H₂O₂ to stimulate JNK activation. Cell extracts were incubated with c-Jun fusion protein beads to pull down JNK. Kinase reactions were performed and c-Jun phosphorylation was assessed by Western blotting as described in the Materials and methods section. Band intensities were assessed by densitometry and expressed relative to untreated controls. Results represent the means for three independent experiments. Significant differences from untreated controls are designated as ** ($P < 0.01$) or *** ($P < 0.001$). Significant differences from similarly treated pre-confluent cells are designated as ††† ($P < 0.001$).

Figure 3 GSTA1-JNK complex formation is increased in post-confluent Caco-2 cells and unaffected by cellular stresses

Pre-confluent (Pre) and post-confluent (Post) Caco-2 cells were exposed to H₂O₂, IL-1 β or UV irradiation to stimulate JNK activation. Cell extracts were incubated with c-Jun fusion protein beads to pull down JNK and then examined by Western-blot analysis using antibodies to GSTA1-1. Band intensities were assessed by densitometry and expressed relative to untreated controls. Results are the means for three independent experiments. ***Significantly different from control ($P < 0.001$).

cell extracts of pre-confluent and post-confluent cells exposed to H₂O₂, IL-1 β or UV irradiation (Figure 3). Densitometric analysis revealed that, compared with pre-confluent cells, GSTA1-1 protein bound to JNK protein was 4.0-, 4.2-, 4.9- and 3.3-fold higher ($P < 0.001$) in post-confluent cells that were either untreated or treated with H₂O₂, IL-1 β or UV irradiation respectively. However, compared with corresponding controls, the stress treatments did not alter the degree of JNK-GSTA1-1 complex formation in either pre-confluent or post-confluent cells.

Figure 4 GSTA1 overexpression suppresses JNK activation by H₂O₂ and UV irradiation

GSTA1 expression was controlled using the Tet-Off system by culturing MEF3T3-GSTA1-1 cells in the absence (+ GSTA1) or presence (-GSTA1) of doxycycline. To stimulate JNK activation, cells were exposed to (A) H₂O₂ or (B) UV irradiation. Cell extracts were incubated with c-Jun fusion protein beads to pull down JNK. Kinase reactions were performed and c-Jun phosphorylation was assessed by Western blotting as described in the Materials and methods section. Band intensities were assessed by densitometry and expressed relative to untreated controls. Results represent the means for three independent experiments. Significant differences from untreated controls are designated as *** ($P < 0.001$). Significant differences from similarly treated GSTA1-expressing cells are designated as †† ($P < 0.01$) or ††† ($P < 0.001$).

Overexpression of GSTA1 in MEF3T3 cells suppresses JNK activation

In order to more specifically confirm the role of GSTA1 in modulating JNK activation in response to stress stimuli, we developed a Tet-Off-based GSTA1-1-inducible MEF3T3 cell line to control GSTA1 expression. JNK activity was monitored in MEF3T3-GSTA1-1 cells maintained in the presence or absence of doxycycline as described in the Materials and methods section. Removal of doxycycline resulted in a 5.6 ± 0.1 -fold increase ($P < 0.05$) in expression of GSTA1 by 24 h. In MEF3T3-GSTA1-1 cells expressing low constitutive GSTA1-1 activity (doxycycline present), JNK activation rose 23.5-fold ($P < 0.001$) 1 h following exposure to H₂O₂ (Figure 4). By 3 h post-exposure to H₂O₂ the degree of increase in JNK activity decreased to 15.6-fold ($P < 0.001$). JNK activation was significantly less ($P < 0.001$) in MEF3T3-GSTA1 cells that overexpress GSTA1 (doxycycline absent) with increases of 11-fold ($P < 0.001$) and 8.5-fold ($P < 0.001$) at 1 h and 3 h following H₂O₂ exposure respectively. Exposure to UV light resulted in a 5.3-fold increase ($P < 0.001$) in JNK activity in MEF3T3-GSTA1-1 cells with low GSTA1 expression; however, there was no significant change in JNK activity in MEF3T3-GSTA1-1 cells that overexpress GSTA1.

We also compared the effect of IL-1 β , H₂O₂ and UV light on functional activation of c-Jun by phosphorylation in MEF3T3-GSTA1-1 cells with or without GSTA1 expression using the PathDetect c-Jun *trans*-Reporting System as described in the Materials and methods section. In MEF3T3-GSTA1-1 cells with low GSTA1 activity, treatment with IL-1 β , H₂O₂ and UV light resulted in 1.9-fold ($P < 0.001$), 1.6-fold ($P < 0.01$) and 1.5-fold ($P < 0.05$) increases in luciferase activity, indicating activation/phosphorylation of c-Jun (Table 1). There was no significant increase in luciferase activity in MEF3T3-GSTA1-1 cells that overexpress GSTA1 following exposure to IL-1 β ,

Table 1 GSTA1 expression abrogates c-Jun phosphorylation in response to various cellular stresses

MEF3T3-GSTA1 cells were transiently transfected with c-Jun-GAL4, pFR-Luc and *Renilla* luciferase as described in the Materials and methods section. GSTA1-1 expression was controlled using the Tet-Off system by culturing MEF3T3-GSTA1-1 cells in the presence (–GSTA1-1) or absence (+GSTA1-1) of doxycycline. Cells were exposed to IL-1 β , H₂O₂ or UV irradiation to stimulate JNK activation. Luciferase activities (means $\pm$ S.E.M.) from three independent experiments are expressed relative to control values from untreated cells without GSTA1 expression. Significant differences from control values of GSTA1-deficient cells are designated as * ($P < 0.05$), ** ($P < 0.01$) or *** ($P < 0.001$).

Treatment	–GSTA1-1	+GSTA1-1
Control	1.00 $\pm$ 0.29	0.72 $\pm$ 0.04
IL-1 β	1.87 $\pm$ 0.29***	0.83 $\pm$ 0.09
H ₂ O ₂	1.59 $\pm$ 0.06**	0.67 $\pm$ 0.02
UV	1.48 $\pm$ 0.03*	0.31 $\pm$ 0.01*

Table 2 TNF- α /butyrate-induced apoptosis is reduced in post-confluent Caco-2 cells

Cells were treated with TNF- α /butyrate for 24 or 48 h, counterstained with DAPI and assessed for apoptosis by TUNEL or by immunofluorescent labelling of active caspase 3 as outlined in the Materials and methods section. Data represent the incidence of apoptosis (means $\pm$ S.E.M.) compared with untreated control cells from three separate experiments. Significant differences from untreated controls are designated as *** ($P < 0.001$). Significant differences from treated pre-confluent cells are designated as ††† ($P < 0.001$). n.d., not determined.

Duration of exposure	TUNEL		Active caspase 3	
	Pre-confluent	Post-confluent	Pre-confluent	Post-confluent
24 h	25 $\pm$ 0.16***	4.2 $\pm$ 0.4***†††	12.1 $\pm$ 1.8***	2.1 $\pm$ 0.3†††
48 h	46 $\pm$ 2.0***	29 $\pm$ 2.0***†††	n.d.	n.d.

H₂O₂ and UV irradiation. Indeed, luciferase activity was reduced ($P < 0.05$) in GSTA1-expressing cells exposed to UV irradiation.

JNK-mediated apoptosis is reduced in post-confluent cells expressing high levels of GSTA1

To establish the functional significance of GSTA1-1-mediated inhibition of JNK activation, we assessed the incidence of apoptosis in pre-confluent and 7 days post-confluent Caco-2 cells exposed to TNF α and butyrate for either 24 or 48 h as described in the Materials and methods section. At 24 h, TUNEL analysis revealed a 25 % incidence ($P < 0.001$) of apoptosis in populations of pre-confluent cells, whereas only 4.2 % ($P < 0.001$) of post-confluent cells were apoptotic (Table 2). Caspase 3 was activated in 12.1 % of pre-confluent cells ($P < 0.001$); however, only 2.1 % of post-confluent cells had evidence of activation. Based on these analyses, the incidence of apoptosis was 6-fold greater ($P < 0.01$) in pre-confluent Caco-2 cells compared with post-confluent cells. At 48 h, 46 % of pre-confluent cells were apoptotic, whereas the incidence of apoptosis in post-confluent cells was 29 % as assessed by TUNEL analysis.

DISCUSSION

In the present study, we demonstrate a role for GSTA1 in modulating JNK activity in response to various cellular stresses including a pro-inflammatory cytokine and oxidative stress. Whereas direct inhibitory interactions between GSTP and JNK [3,16], and between GST Mu and ASK1 [13], have been shown previously, involvement of human GSTA1 in the regulation of stress signalling and JNK activation has not yet been identified. Following activation by stress signals, JNK participates in the

regulation of numerous cellular processes including proliferation, differentiation, apoptosis and DNA repair. It is evident from the results of the present study that the level of GSTA1 expression establishes the degree of JNK responsiveness to stress stimuli and the propensity to undergo apoptosis.

We chose to investigate the influence of GSTA1 overexpression on JNK activity in Caco-2 cells because of a postulated involvement of GSTA1 in control of cellular differentiation. When cultured to confluency, Caco-2 cells spontaneously differentiate into an enterocyte-like phenotype characterized by cell polarization, development of apical microvilli, appearance of intercellular tight junctions and increased expression and activity of brush border enzymes [17,18]. Our demonstration of progressive increases in GSTA1 expression levels at later stages of cell confluency confirms previous findings of GSTA1 overexpression in differentiated Caco-2 cells [19]. Similarly, reduced GSTP1 expression in rat hepatocytes is associated with proliferation in contrast with overexpression during differentiation [20,21]. The biological relevance of GSTA1 induction with increasing confluency has been attributed to a 'sensor' capacity to respond to changes in redox potential during oxidative stress, representing a critical determinant of cellular differentiation and apoptosis [19]. While Alpha class GST genes, notably GSTA1, GSTA2 and GSTA4, have the capacity to protect against oxidative stress through detoxification of products of lipid peroxidation, our findings and the results of studies on *hGSTA2* and *mGsta4* [10,12] suggest that Alpha class GSTs also participate in the regulation of cellular stress responses.

The progressive increase in GSTA1-1 levels observed in differentiating Caco-2 cells were associated with reduced JNK phosphorylation of c-Jun following stress stimulation. While comparison of GSTA1 expression and JNK activity in Caco-2 cells implicates GSTA1 in stress signalling by association, other factors, possibly other GST classes, may also play a role. However, immunoprecipitation experiments confirmed that interactions with JNK are enhanced as GSTA1 expression increases in post-confluent cells. Collectively, these data suggest that, by regulating JNK signalling, GSTA1 expression may control cellular events that govern differentiation. Thus GSTA1-1-mediated control of JNK activation by oxidative stress and pro-inflammatory cytokines may be critical in maintaining the appropriate balance between proliferating and differentiated enterocytes in the colonic mucosa.

By controlling GSTA1 expression in MEF3T3 cells using the Tet-Off system we demonstrated that overexpression significantly reduced JNK activation in response to IL-1 β , UV irradiation and H₂O₂ compared with low level GSTA1 expression. This confirms the involvement of GSTA1-1 in JNK inhibition and implicates this isoenzyme in reducing the magnitude of JNK responses to a variety of cellular stresses. A similar approach conducted in NIH3T3 cells showed that overexpression of GSTP1 reduced JNK activation in non-stressed cells and significantly reduced the degree of H₂O₂-induced JNK activation relative to changes seen in the absence of GSTP1 [8]. Additionally, JNK activation was significantly reduced in *hGSTA2-2*-overexpressing human erythroleukaemia cells (K562) exposed to H₂O₂ [12]. Previous studies have shown that GSTP1/2-mediated inhibition of JNK is due to direct interactions that dissociate in the presence of oxidative stress [3]. Moreover, studies in mouse fibroblasts (3T3-4A) have shown that increased levels of ROS decrease JNK inhibition by reducing GST–JNK complex formation [3]. However, our results did not demonstrate any reductions in GST–JNK interactions following exposure to cellular stressors.

In his seminal study, Adler et al. [3] demonstrated that GST Pi, Mu and Alpha classes are all capable of inhibiting JNK to

varying degrees with Alpha being the least potent inhibitor. A subsequent study demonstrated that JNK and mouse GSTA4 co-immunoprecipitate but effects on JNK activity were not examined [22]. It is plausible that GST class-specific inhibition of JNK may establish the specific cellular processes that are regulated. Indeed, *GSTP1* expression in rat hepatocytes is predominantly associated with cellular proliferation, whereas *GSTA1/2* relates more to differentiation [20].

To evaluate the biological implications of GSTA1-1-mediated suppression of JNK in Caco-2 cells, we assessed the prevalence of apoptosis in pre- and post-confluent Caco-2 cells treated with TNF α and sodium butyrate. Butyrate is a short-chain fatty acid produced by bacterial fermentation of complex carbohydrates that, at low doses, causes enterocyte differentiation and, at higher doses, induces apoptosis [23]. Furthermore, apoptosis in Caco-2 cells treated with butyrate is JNK-dependent [24] and is mediated through activation of the caspase cascade [25]. In this way, butyrate-mediated apoptosis in Caco-2 cells acts as an indicator of JNK activation. We demonstrated a 6-fold higher incidence of butyrate-induced apoptosis in pre-confluent Caco-2 cells that express GSTA1-1 at low levels compared with post-confluent cells that overexpress *GSTA1*. Along with the capacity of GSTA1-1 to inhibit JNK, this finding may explain, in part, why butyrate readily induces apoptosis in undifferentiated colon cancer cells and to a much lesser degree in differentiated cells [23]. Other studies have also supported a role for GSTs in the prevention of apoptosis. Increased expression of *GSTP1* protects against dopamine-induced apoptosis in dopaminergic neurons by decreasing JNK activity [9]. In addition, transfection of *GSTM1*, *GSTA1* or *GSTP1* abolishes caspase 3 activity and reduces apoptosis in rat liver and overexpression of *hGSTA2-2* protects against apoptosis in K562 cells [12,26]. Interestingly, thioacetamide-mediated apoptosis in rat liver is dependent on JNK and caspase 3 activation as well as marked reductions in *GSTA1/2* and *GSTM1/2* expression [26]. Moreover, DMSO protects against thioacetamide-mediated apoptosis by preventing *GSTM1/2* and *GSTA1/2* down-regulation. Collectively, these findings illustrate a key role for GSTs in controlling apoptosis in various cell types.

Apoptosis is considered to be a normal consequence of enterocyte terminal differentiation *in vivo*. As enterocytes migrate from the colonic crypts to the luminal surface they progressively cease to proliferate, differentiate, undergo apoptosis and slough into the intestinal lumen [27]. Levels of apoptosis must be balanced with cellular proliferation to control tissue hyperplasia or regression. Thus control of enterocyte apoptosis is necessary to maintain barrier function of the intestinal epithelium and to avert the development of villus atrophy or neoplastic transformation. Indeed, previous studies have suggested that the duration of JNK activation may determine cellular responses with transient activation leading to cell proliferation/differentiation as opposed to sustained activation resulting in apoptosis [28,29]. The relationship between elevated GST expression and resistance to apoptosis [30] and the consequences of abnormal regulation of cellular differentiation and apoptosis underscore the critical role that GSTs play in modulating JNK signalling pathways [31].

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