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Transcriptional control of glyoxalase 1 by Nrf2 provides a stress responsive defence against dicarbonyl glycation

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Running title: Transcriptional control of glyoxalase 1 by Nrf2

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SYNOPSIS

Abnormal cellular accumulation of the dicarbonyl metabolite methylglyoxal occurs on exposure to high glucose concentration, inflammation, cell ageing and senescence. It is associated with increased methylglyoxal-adduct content of protein and DNA linked to increased DNA strand breaks and mutagenesis, mitochondrial dysfunction and reactive oxygen species formation and cell detachment from the extracellular matrix. Methylglyoxal-mediated damage is countered by glutathione-dependent metabolism by glyoxalase-1. It is not known, however, if glyoxalase-1 has stress responsive up-regulation to counter periods of high methylglyoxal concentration or dicarbonyl stress. We identified a functional antioxidant response element in the 5'-untranslated region of exon-1 of the mammalian glyoxalase-1 gene. Transcription factor Nrf2 binds to this antioxidant response element increasing basal and inducible expression of glyoxalase 1. Activators of Nrf2 induced increased glyoxalase-1 mRNA, protein and activity. Increased expression of glyoxalase-1 decreased cellular and extracellular concentrations of methylglyoxal, methylglyoxal-derived protein adducts, mutagenesis and cell detachment. Hepatic, brain, heart, kidney and lung glyoxalase-1 mRNA and protein were decreased in Nrf2 (-/-) mice and urinary excretion of methylglyoxal protein and nucleotide adducts were increased *ca.* 2-fold. We conclude that dicarbonyl stress is countered by up-regulation of glyoxalase-1 in the Nrf2 stress responsive system, protecting protein and DNA from increased damage and preserving cell function.

Key words: Nrf2, glyoxalase, methylglyoxal, DNA damage, protein damage, glycation.

Abbreviations used: AITC, allyl isothiocyanate; ARE, antioxidant response element; CDDO-Me, methyl 2-cyano-3,12-dioxo-oleana-1,9(11)dien-28-oate; ChIP, chromatin immunoprecipitation; CNC, cap 'n' collar; ECL, enhanced chemiluminescence; Glo1, glyoxalase 1; keap1, kelch (β -propeller tertiary structure)-like erythroid cell-derived protein with CNC homology-associated protein 1; IRE, insulin response element; MG, methylglyoxal; MGdG, 3-(2'-deoxyriboseyl)-6,7-dihydro-6,7-dihydroxy-6/7-methylimidazo-[2,3-b]purin-9(8)one; MG-H1, N δ -(5-hydro-5-methyl-4-imidazolone-2-yl)-ornithine; Nrf2, nuclear erythroid factor E2 related factor-2; ROS, reactive oxygen species; SFN, sulforaphane; TBST, tris-buffered saline with Tween-20.

INTRODUCTION

Modification of proteins and DNA by the dicarbonyl metabolite methylglyoxal (MG) has emerged as an important endogenous threat to the functional integrity of the proteome and genome. MG is formed by the spontaneous degradation of triosephosphate intermediates and is an unavoidable by-product of anaerobic glycolysis [1]. MG reacts with proteins and DNA forming quantitatively major adducts of endogenous damage, similar to and in some cases exceeding the steady-state levels of adducts produced by oxidative damage. Modification of proteins by MG is directed to arginine residues forming the hydroimidazolone, N_δ-(5-hydro-5-methyl-4-imidazolone-2-yl)-ornithine (MG-H1) and modification of DNA is directed to deoxyguanosine residues forming the imidazopurinone 3-(2'-deoxyriboseyl)-6,7-dihydro-6,7-dihydroxy-6/7-methylimidazo-[2,3-b]purin-9(8)one (MGdG) [2;3] (Figure 1, A and B). MG modification of DNA increases DNA strand breaks and frameshift mutation [3;4]. Protein modification by MG is directed to functional sites where it is associated with metabolic, structural and functional abnormalities: for example, mitochondrial dysfunction with increased the formation of reactive oxygen species (ROS) [5], cell detachment from the extracellular matrix by decreased integrin binding to MG-modified extracellular matrix proteins and anoikis [6;7], and induction of accelerated cell senescence [8]. Protein and DNA damage by MG is suppressed by glyoxalase 1 (Glo1) which catalyses the GSH-dependent conversion of MG to S-D-lactoylglutathione. Further metabolism of S-D-lactoylglutathione to D-lactate is catalysed by glyoxalase 2 which restores GSH consumed in the Glo1-catalysed reaction [9] (Figure 1C). The high reactivity of MG leads to some escape from this detoxification such that in cells 1 – 5% of proteins contain MG-H1 residues and up to 1 in 10⁵ nucleotides in DNA is a MGdG adduct [2;3].

Protein and DNA damage increases in periods of increased MG formation and decreased metabolism – such as metabolic stress associated with increased glucose metabolism [6], decreased GSH in oxidative stress and GSH conjugation with xenobiotic compounds [10], inflammatory signalling [11] and ageing and senescence [12;13]. In such conditions a stress responsive up-regulation of expression of the Glo1 gene, *GLO1*, would seem to be advantageous although no such regulatory mechanism is currently known.

Nuclear erythroid factor E2 related factor-2 (Nrf2) is a member of the cap 'n' collar (CNC) subfamily of basic-region leucine zipper transcription factors. It is an essential transactivator of genes containing one or more antioxidant response elements (AREs) in their regulatory regions. Nrf2 coordinates increased expression of a battery of ARE-linked genes associated with protection against oxidative stress and electrophilic metabolites and decreased expression of lipogenic genes for cell survival and defence [14]. Under basal conditions, Nrf2 is complexed with a Kelch (β-propeller tertiary structure)-like erythroid cell-derived protein with CNC homology-associated protein 1 (keap1). keap1 is a substrate adaptor protein for Cullin-3-dependent E2 ubiquitin ligase complex, directing Nrf2 for degradation by the 26S proteasome [15;16].

In this report we describe discovery of a functional ARE in exon-1 of *GLO1* which serves to engage Glo1 in the Nrf2 stress responsive transcriptional system. MG, Glo1 and dicarbonyl stress now emerge as a downstream target by which the Nrf2/Keap1 system exerts its protective functions.

EXPERIMENTAL

Cell culture and treatments

Human hepatoma HepG2 cells and human BJ fibroblasts were cultured in MEM medium with 10% fetal calf serum and 2 mM glutamine under an atmosphere of 5% CO₂ in air, 100% humidity and 37°C. Activity of Glo1 was determined by measuring the initial rate of

isomerisation of the hemithioacetal formed from MG and GSH to S-D-lactoylglutathione followed spectrophotometrically at 240 nm [17]. The MG content of cells and culture medium was determined by stable isotopic dilution analysis liquid chromatography with tandem mass spectrometric detection [6]. The MG-H1 content of cell protein and the MG-H1 and MGdG contents of mouse urine were determined as described [3;18].

For cell adhesion assays, BJ fibroblasts were incubated with and without 2 μ M sulforaphane (SFN) for 24 h. SFN was non-toxic and nor inhibited cell growth under these conditions – see Supplementary data. The culture medium was then removed, incubated with human type IV collagen for 24 h and human microvascular endothelial HMEC-1 cell attachment studied as described [6].

Frameshift mutation analysis

The pEGFP-CA/T12 vector is a reporter for efficiency of transfection while pEGFP-CA13 vector is a reporter of frameshift mutation[19]. HepG2 cells (2×10^5 cells/well) were cultured in 12-well plates for 24 h, the medium removed, the cells washed with serum free medium and replaced with DNA/lipofectamine complexes in serum free medium (Lipofectamine 2000 reagent, 2 μ l; 0.8 μ g pEGFP-CA13 or pEGFP-CA/T12; 2.0 ml serum free medium) and incubated for 5 h at 37^oC. The transfection medium was removed and replaced with complete medium (MEM supplemented with 10% FCS and 2 mM glutamine) and incubated for 48 h. Transfection efficiency was assessed quantitatively by flow cytometry. For HepG2 cells *in vitro*, the median growth inhibitory concentration of MG was 1.07 ± 0.08 mM (n = 8). HepG2 cells transfected with expression of pEGFP-CA/T12 were incubated with or without 2 μ M AITC for 24 h and then with or without 400 μ M MG for a further for 48 h. Thereafter, cells were washed with PBS and analysed for GFP expression by flow cytometry.

Real-time PCR

Total RNA was extracted from cultured cells which were treated with SFN using RNeasy Mini Kit (QIAGEN). cDNAs were synthesized with oligo (dT)18 primer and BioScript reverse transcriptase (BIOLINE). Human *GLO1* and *ACTB* (β -actin reference gene) mRNA were quantified by real-time RT-PCR SYBR green method on ABI 7500 fast real-time PCR system. The following primers were used to amplify *GLO1*, forward 5'-ATGCGACCCAGAGTTACCAC-3' and reverse 5'-CCAGGCCTTTCATTTTACCA-3'. The reference gene *ACTB* was amplified with primers 5'-GGACTTCGAGCAAGAGATGG-3' (forward) and 5'-AGCACTGTGTTGGCGTACAG-3'(reverse). For mouse *GLO1*, the following primers were used: forward primer sequence 5'-GATCCAGACCCTAGCACCAA-3' and reverse sequence is 5'-CTTCTGCAGGAGGGTCAGTC-3'. The reference gene was *18s rRNA* with primers purchased from Qiagen. The efficiency of silencing by Nrf2 siRNA was assessed 24 h post-treatment by measurement of decrease in Nrf2 mRNA.

Western blotting analysis

Protein extracts (30 μ g) were subjected to SDS-PAGE on 10% polyacrylamide gels. After electrophoresis, the proteins were transferred electrophoretically to PVDF membrane and the membrane blocked with 5% non-fat milk in tris-buffered saline with Tween-20 (TBST) buffer (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.05% Tween-20) and probed with rabbit anti-human Glo1 antibody.[17] The membrane was incubated at 4^oC overnight. After washing, the membrane was incubated with horseradish peroxidase conjugate second antibody for 1 h at room temperature. Immunoreactivity was detected with enhanced chemiluminescence (ECL) and intensities of protein bands were quantified by software ImageQuant TL (GE Healthcare). For reference protein, β -actin, the membrane was stripped with stripping buffer (100 mM β -mercaptoethanol, 2% SDS, 62.5 mM Tris-HCl pH 6.8),

blocked with 5% non-fat milk in TBST buffer and re-probed with anti- β -actin antibody with ECL detection.

Construction of Glo1-ARE and related reporter plasmids

pGL3-NQO1ARE - Double-stranded DNA oligomer containing the quinone reductase (*NQO1*) ARE was inserted into pGL3-basic vector by Kpn1 and Nhe1 double digestion to construct an ARE positive reporter vector, pGL3-NQO1ARE. The insertion sequence was (ARE motif is underlined and low case letters are the Kpn1 and Nhe1 restriction sites): ggtaccGAGTCGGAAGGTTTAGGCGTCAGTGTCAGTCTTAGcgatcg. *GLO1 promoter pGL3-basic reporter vector construction* – A *GLO1* gene fragment extending from -1 (the first base before start codon) to -1176 containing 3 putative AREs and a serial deletion fragments were amplified by PCR from human genomic DNA and cloned into pGL3-basic reporter vector. The primers were used to create deletion mutants of human *GLO1* promoter fragments as listed in Table 1. Kpn1 and Nhe1 restriction sites were added in PCR primers 5' and 3' ends. PCR fragments of the 5'-flanking region in *GLO1* gene were amplified using human genomic DNA, and cloned into pJET1.2 Cloning Vector with ClonJET™ PCR cloning kit (Fermentas). The pJET1.2 *GLO1* promoter vector was digested with Kpn1 and Nhe1 and the fragments of *GLO1* promoter region were sub-cloned into pGL3-basic vector. For the mutant ARE-1, a mutant anti-sense primer (see Table 1) was used to amplify the 5'-flanking region of *GLO1* promoter and sub-cloned to pGL3-basic reporter vector. All insertion sequences were confirmed by DNA sequence analysis. Four wild-type and two mutant type pGL3 reporter vectors for *GLO1* promoter were constructed. To assess the conservation of ARE1 across species, Glo1 exon-1 DNA sequence was aligned to the human counterpart by ClustalW2 (EMBL-EBI).

Transfection and luciferase assay of custom vectors

For luciferase assays, 2×10^5 HepG2 cells per well were plated into 24-well plates and cultured overnight. The cells were transfected with 0.5 μ g reporter vector and 10 ng pRL-TK plasmid using Lipofectamine 2000 according to manufacturer's protocol. The empty pGL3-basic vector was used as control. After 24 h, 4 μ M SFN was added to the cells and vehicle (DMSO) added to control. After 24 h, the cells were washed with PBS and luciferase activity assay performed immediately or samples stored at -80 °C until analysis. For the reporter assay, 100 μ l Cell Culture Lysis Reagent (CCLR, Promega) was added to cell extracts and shaken gently for 30 min. The activity mixture was centrifuged (12,000g, 5 min, 4 °C) and an aliquot (20 μ l) of supernatant used in the reporter assay. The luciferase activity was determined using a Dual Luciferase Reporter Assay System (Promega). The relative luciferase activities were normalized by co-transfection of pRL-TK vector. The cell viability in the transfection assays was 91 – 96 %.

Chromatin immunoprecipitation

Chromatin immunoprecipitation (ChIP) was performed using EZ-ChIP kit (Merck-Millipore). HepG2 cells (3×10^6 ; seeding density 52,000 per cm^2) were incubated for 24 h and then treated with 4 μ M SFN or vehicle (0.004% DMSO) for 4 h. Formaldehyde (1%) was added to the cells and samples incubated at room temperature for 10 min to crosslink protein-DNA complexes. Excess formaldehyde quenched by incubation with glycine (62.5 mM) for 5 min at room temperature. Cells were washed twice with PBS containing protease inhibitor cocktail II and cells collected. Cell pellets were re-suspended with 1 ml SDS lysis buffer and sonicated (15 x 20 s, 25% output) and centrifuged (12,000g, 4°C, 10 min). An aliquot of supernatant (100 μ l) was diluted with 900 μ l ChIP dilution buffer and non-specific

immunoglobulin binding activity removed by incubation with protein G-agarose for 1 h. Target DNA fragments were then immunoprecipitated by addition of anti-Nrf2 antibody (H-300, 5 µg; Santa Cruz Biotechnology) incubated overnight at 4°C. Anti-RNA polymerase II antibody and non-immune IgG (1 µg) were used for controls. Antibody-protein-DNA complex was collected by adding protein G-agarose. After washing with immune complex wash buffers, DNA was released by incubation of samples at 65 °C overnight and mRNA protein degraded by addition of RNase and proteinase K. Enriched DNA was purified by microspin columns (ChIP DNA clean & Concentrator Kit, Zymo Research, Irvine, USA). PCR and real-time PCR were performed with following primers designed based on the GLO1 promoter target region, forward 5'-GCGTAGTGTGGGTGACTCCT-3' and reverse 5'-TGTGCCACCTTGGTACTG-3'. DNA extract (4 µl) was used as general PCR template with PCR conditions: 94°C for 3 min, 40 cycles denaturation at 94°C for 30 s, annealing at 55 °C for 20 s and extension for 20 s. PCR products were detected with 4% agarose gel and observed by ethidium bromide under UV illumination. For real-time PCR, DNA extract (2 µl) was used as template with SYBR green detection. PCR was performed at ABI 7500 fast real time PCR system at standard PCR condition for 50 cycles. The results were analysed with $\Delta\Delta$ Ct method.

Animal studies

Urine was collected over 24 h under mineral oil from 10-week old wild-type and Nrf2 (-/-) mice and MG adducts, MGdG and MG-H1, and creatinine determined. Other mice were sacrificed, livers removed and snap-frozen immediately in liquid nitrogen, stored at -80 °C and shipped to the collaborating laboratory for Glo1 mRNA and protein analysis. Experimental procedures were approved by the Institutional Animal Experiment Committee of Tohoku University, and experiments were carried out in accordance with the Regulation for Animal Experiments of Tohoku University, Japan or were undertaken in accordance with criteria outlined in a license granted under the Animals (Scientific Procedures) Act 1986, and approved by the Animal Ethics Committees of the University of Liverpool. Generation of the Nrf2 knockout mouse and genotyping of progeny have been described elsewhere [20]. Male mice of approximately 10 weeks of age were used throughout the study. Mice were housed at a temperature range of 19 – 23 °C under 12-h light/dark cycles and given free access to food and water. Animals were killed by exposure to a rising concentration of CO₂ followed by cervical dislocation. Livers were removed and snap-frozen immediately in liquid nitrogen, before being stored at -80 °C. For experiments of treatment with methyl 2-cyano-3,12-dioxooleana-1,9(11)dien-28-oate (CDDO-Me), male wild type and Nrf2 knockout mice (10-12 weeks old) were treated with a single i.p. injection of 3 mg/kg CDDO-Me or diethyl sulfoxide vehicle control (total volume 100 µl). After 24 h the mice were sacrificed and their livers removed and snap frozen in liquid nitrogen, before being stored at -80°C until analysis. CDDO-Me was synthesized in-house using a modified version of the method of Sporn *et al.* [21].

Statistical analysis

Data are mean ± SD for parametric data and median (upper – lower quartile) for non-parametric data. Significance of difference between mean changes was assessed by Student's *t* test and median changes by Mann Whitney-U test. All replicates are independent experiments.

RESULTS

A functional antioxidant response element in exon-1 of human glyoxalase 1 gene

We analysed the 5'-flanking region of the human *GLO1* at locus 6p21.2 for the presence of stress response related regulatory elements. The human *GLO1* gene promoter contains functional insulin response and metal response elements [22]. We also found three AREs located at nucleotide sequence (numbered from the start codon): -10 to -19, sequence 5'-GTGATACTGCA-3' in exon-1 (ARE-1); -261 to -252, sequence 5'-ATGAGTTTGCC-3' (ARE-2); and -1060 to -1051, sequence 5'-ATGACTAAGCC-3' (ARE-3). We explored functionality of these AREs by construction of luciferase reporter vectors with whole or segments of this region. Stimulation of the of Nrf2 system with SFN, an activator of Nrf2 [23], showed that ARE-1 alone gave maximal induction of Glo1 transcriptional response and ARE-2 and ARE-3 had little further effect on the inducible transcriptional response (Figure 1D). Mutation of the ARE-1 blocked the transcriptional response (Figure 1E), indicating that there is inducible expression of *GLO1* by activators of Nrf2.

Binding of Nrf2 to the ARE of exon-1 in human glyoxalase-1 gene - chromatin immunoprecipitation assay

Evidence that *GLO1* ARE-1 was bound by Nrf2 when activated was sought by a ChIP study with anti-Nrf2 antibody. PCR products overlapping the ARE-1 sequence located -9 to -19 of *GLO1* were amplified from immunoprecipitates of HepG2 cells treated with 4 μ M SFN. This gave a strong band whereas corresponding amplification from vehicle-treated cells gave a weak response (Figure 1F). Real-time qPCR showed that Nrf2 antibody-enriched DNA fragments in SFN treated cells were increased two-fold compared to vehicle-treated controls (Figure 1G). This confirms that when activated, Nrf2 binds exon-1 of human *GLO1* and thereby provides a stress responsive inducible increase in Glo1 expression for increased protection against dicarbonyl-mediated protein and DNA damage.

Induction of glyoxalase expression by hydrogen peroxide-induced oxidative stress

Nrf2 coordinates protective gene expression to counter damaging effects of oxidative stress. To assess if the expression of Glo1 is induced by activation of Nrf2 in the ARE transcriptional response, we studied the effect of hydrogen peroxide-induced oxidative stress on the quinone reductase-ARE (NQO1-ARE) transcriptional response – as a positive control [24], and the concomitant glyoxalase 1-ARE (*GLO1*-ARE-1) transcriptional response. HepG2 cells were transfected with NQO1-ARE or *GLO1*-ARE-1 luciferase reporter vectors, with empty vector and mutant glyoxalase 1-ARE-1 (*GLO1*-ARE-1m) as controls, and incubated with 500 μ M hydrogen peroxide to induce oxidative stress. Due to the high constitutive cellular activity of hydrogen peroxide detoxification in HepG2 cells, a relatively high concentration of hydrogen peroxide was required for Nrf2 activation – as found by others [24]; incubation of cells with 50 μ M hydrogen peroxide, for example, gave no increase in NQO1-ARE transcriptional response (data not shown). Reporter assay outputs indicated hydrogen peroxide-induced oxidative stress activated both the NQO1-ARE and *GLO1*-ARE-1 transcriptional responses (Figure 1H), indicating that expression of Glo1 may be increased in oxidative stress via the Nrf2/ARE transcriptional response.

Induction of glyoxalase 1 expression by activators of Nrf2 and protection from dicarbonyl glycation

Activators of Nrf2 are dietary isothiocyanates such as SFN and allyl isothiocyanate (AITC) [25] but responses induced by them have not hitherto be linked to induction of Glo1 expression and decreased DNA and protein damage caused by MG. Responses of Glo1 expression were studied in human hepatoma HepG2 cells and BJ fibroblasts *in vitro*. Treatment with 2 μ M SFN or AITC increased the activity of Glo1 2 – 3 fold (Figure 2A and

2B). There was a related dose-dependent increase in Glo1 mRNA and time course changes showing increase to 12 h post-treatment with inducer and decline back to baseline levels thereafter (Figure 2C and 2D). Assessment of Glo1 protein showed a progressive increase over 16 h (Figure 2E). The involvement of Nrf2 in induction of Glo1 expression was assessed by siRNA silencing of Nrf2. The efficiency of silencing by transfection with Nrf2 siRNA, as judged by decrease in Nrf2 mRNA normalised to β -actin mRNA housekeeping reference, was 78 ± 9 %. Silencing of Nrf2 expression with siRNA decreased the basal mRNA of Glo1 and blocked the increase of Glo1 mRNA in response to SFN (Figure 2F). At 24 h post-treatment there was decreased concentration of MG in the cell culture medium and cells (Figure 2G and 2H), and related decrease in cellular protein MG-H1 residue content developed after 48 h (Figure 2I). Activation of Nrf2, therefore, increased Glo1 expression and activity, decreasing cellular and extracellular concentrations of MG leading to decreased damage to cellular protein.

Functional effects of induction of GLO1 expression – decreased mutagenesis and loss of cell-matrix interaction

We examined if increased expression of Glo1 was associated with resistance to cell functional impairment mediated by methylglyoxal, taking MG-mediated mutagenesis and cell detachment from extracellular matrix as examples.

To assess the effect of inducible expression of Glo1 on MG-induced mutagenesis, we employed a GFP fluorogenic frameshift mutation reporter developed by Kirsch and co-workers [26] and previously used to study frameshift mutation in oxidative stress [19]. Using this we found that MG-induced increased frameshift mutations were prevented by prior induction of Glo1 expression by 2 μ M AITC (Figure 3A). Increased formation of MG by cells leads to increased release of MG into the extracellular fluid or culture medium [6]. Increased extracellular concentration of MG increases modification of arginine residues in integrin binding sites of extracellular matrix proteins and decreases cell-extracellular matrix interactions leading to cell detachment and anoikis – as found for vascular endothelial cells [6], renal mesangial cells [7] and peripheral neurones [27]. Induction of Glo1 expression in BJ fibroblasts decreased extracellular MG – see above. Conditioning of type IV collagen in this MG-depleted medium preserved the binding of endothelial cells beyond that found with control culture medium (Figure 3B). These studies show that increased expression of Glo1 by activation of the Nrf2 system counters functional impairment of DNA and extracellular matrix proteins.

Regulation of glyoxalase 1 expression by Nrf2 and protection against protein and nucleotide damage *in vivo*

To assess if this protective mechanism of Nrf2 and Glo1 operates *in vivo* we measured the urinary efflux of nucleoside adduct MGdG and arginine-derived MG-H1 free adduct in wild-type and mutant Nrf2 (-/-) mice. There was decreased expression of Glo1 in mutant Nrf2(-/-) mice, as evidenced by decreased Glo1 mRNA (- 18%, $P < 0.001$) and protein (- 39%, $P < 0.01$) in the liver (Figure 3C and 3D). There were similar decreases of Glo1 protein in the brain, heart and kidney (- 27%, - 30%, and - 26% respectively, $P < 0.001$), and a smaller decrease of Glo1 protein in the lung (- 8%, $P < 0.01$) – Figure 3E – 3H. Wild type Nrf2(+/+) mice treated with the Nrf2 activator CDDO-Me (3 mg/kg) had a small, significant increase in Glo1 protein content of liver in wild type mice (+ 6%, $P < 0.05$) but not in Nrf2(-/-) mice (Figure 3I). We also assessed markers of nucleotide and protein damage in wild type Nrf2(+/+) control mice and mutant Nrf2(-/-) mice. There was *ca.* 2-fold increase in urinary excretions of MGdG and MG-H1 in Nrf2(-/-) mice with respect to wild type Nrf2(+/+) controls, suggesting that the

Nrf2 system does indeed serve to protect against MG-mediated nucleotide and protein damage *in vivo* (Figure 3J and 3K).

DISCUSSION

The glyoxalase system is a detoxification system for endogenous acyclic dicarbonyl metabolites such as MG and glyoxal. It has an established role in the enzymatic defence against protein and nucleotide glycation by dicarbonyls [28] but transcriptional control of Glo1 by a stress responsive mechanism has not hitherto been disclosed. Induction of Glo1 expression has been found in cells exposed to metabolic and inflammatory stress *in vitro* and *in vivo*: glomerular cells challenged by increased MG formation in hyperglycaemia associated with diabetes [29], mesothelial cells challenged by MG and high glucose concentrations of clinical dialysis fluids [30], and inflammatory stress induced by amyloidosis of Alzheimer's disease [31] and overexpression of α -synuclein in an experimental model of Parkinson's disease [32]. In these cases of increased cell dysfunction and damage and physiological morbidity, Glo1 induction was insufficient to prevent increased protein damage by MG. The mechanism of induction was unknown but can now be linked to the endogenous Nrf2-mediated antistress gene response known to be activated in these conditions [33-36]. Studies with Glo1 overexpressing transgenic animals have indicated that increased expression of Glo1 has beneficial effect – maintenance of vascular cell responses, prevention of myocardial cell death when oxygenated after ischaemia and delay of ageing and senescence [13;37;38].

In this study we found that Glo1 mRNA, protein and activity were increased in HepG2 cells and BJ fibroblast *in vitro* on treatment with activators of Nrf2, SFN and AITC, and this was mediated by interaction of Nrf2 with an ARE in exon-1 of the *GLO1* gene. In NJ fibroblasts the Glo1 mRNA was increased by treatment with SFN for up to 12 h and thereafter declined back to control levels by 24 h. There was concomitant increase in Glo1 protein up to 12 h and little decline thereafter at 16 h. These temporal changes are likely due to inactivation of SFN by hydrolysis in the initial 8 - 12 h and induction of Glo1 mRNA and protein where the latter is longer lived than the former. Consistent with this was the hydrolysis and inactivation of the related dietary isothiocyanate phenethyl isothiocyanate with a half-life of ca. 2.4 h under physiological conditions [39] and half-lives of Glo1 mRNA and protein of 15 h (HepG2 cells) and 63 h (MCF7 cells), respectively [40;41].

The antistress gene response mediated by Nrf2 coordinates increased expression of protective genes and decreased expression of lipogenic genes for survival and defence in stressful conditions [14]. Likely critical in achieving this is protection of proteins and DNA from endogenous damaging modifications. It is in keeping with this function, therefore, that activation of Nrf2 increases the expression of Glo1 and protects against dicarbonyl stress. Dicarbonyl stress is an imbalance between formation of dicarbonyl metabolites and enzymatic defences against them in favour of the former leading to increased cell damage. It has emerged as an important endogenous threat to the functional integrity of the proteome and genome [3;18]. Enhanced protection against MG-mediated damage by induction of Glo1 expression is a novel and vital mechanism by which Nrf2-antistress gene response is a guardian of physiological systems (Figure 4A). Increased cellular concentrations of MG were found in experimental models of oxidative stress *in vitro* [10]. Herein we found that hydrogen peroxide-induced oxidative stress activated Nrf2 and increased the ARE-NQO1 transcriptional response - a marker of the protective Nrf2/ARE stress response system[24]. There was a concomitant increase in the Glo1-ARE transcriptional response. This further supports the role of increased expression of Glo1 via the Nrf2 system as a protective stress response which may be activated in oxidative stress.

Proteins susceptible to dicarbonyl modification with related functional impairment - the dicarbonyl proteome – have yet to be fully characterised but they include mitochondrial proteins, extracellular matrix proteins, lipoproteins, transcriptional factors and others [42]. Increased functional impairment is linked to structural impairment of proteins and signalling dysfunction in multiple metabolic pathways [43], indicating its importance and more general significance in physiological systems.

Transcriptional regulation of Glo1 by Nrf2 may provide a new strategy in enhancing Glo1 expression for prevention of cell dysfunction and death in ageing and disease. It also explains previous independent findings of increased Glo1 expression in an experimental background of Nrf2 activation that where the origin and mechanism of this increase was not explored. Induction of Glo1 expression was found in murine intestinal tissue and brain cortex by treatment of mice with butylated hydroxyanisole and the flavonol fisetin, respectively - both compounds are activators of Nrf2 [44;45]. Herein we found a weak induction of murine hepatic Glo1 by the potent Nrf2 activator CDDO-Me in wild type but not in Nrf2 (-/-) mice. To decrease the cellular and extracellular concentrations of MG, induction of Glo1 expression as a pharmacologic strategy for therapeutic intervention has a significant advantage over other approaches – such as chemical scavengers of MG (for example aminoguanidine [46]) - as only a small amount of Glo1 inducer is required to enhance endogenous catalytic removal of MG and the high chemical reactivity necessary for effective MG scavenging agents (and related risk of adverse effects) is not required.

Likely benefits of pharmacological induction of Glo1 are evidenced from studies of overexpression of Glo1 models of disease mechanisms. Experimental evidence is currently best established for vascular complications of diabetes. In cell culture systems incubated in high glucose concentrations to model diabetes associated hyperglycemia, overexpression of Glo1 prevented increased formation of ROS, inflammatory mediators S100A8, S100A12 and high mobility box-1 protein, and increased expression of the receptor for advanced glycation endproducts (RAGE) [47], and corrected decreased angiogenesis of endothelial cells [48] and dysfunction of endothelial progenitor cells [49]. Employing Glo1 transgenic rats, overexpression of Glo1 in vivo decreased the AGE and oxidative damage marker contents of tissues and prevented impairment of endothelium-dependent vasorelaxation of the streptozotocin-induced diabetic rats [37;50]. Recent research has also indicated that Glo1 may protect against formation of atherogenic, small dense low density lipoprotein (LDL) [51]. Glo1 inducers may therefore find use in treatment and prevention of early stage vascular complications of diabetes – nephropathy, retinopathy, peripheral neuropathy and cardiovascular disease [52]. Glo1 has a regulatory insulin response element (IRE) that also regulates expression [22] but treatment with insulin was not sufficient to prevent increased MG and MG-modified protein damage in experimental and clinical diabetes [18;53-55]. A further application for Glo1 inducers is prevention of renal and myocardial reperfusion injury. Glo1 transgenic rats were resistant to renal reperfusion injury, showing decreased AGE accumulation, oxidative stress and renal tubular cell apoptosis [38]. Similar evidence is currently unavailable for myocardial reperfusion injury after acute myocardial infarction but MG has been found to induce increases cardiomyocyte ischemia-reperfusion in vitro [56]. Glo1 inducers are also likely to find benefit in renal failure, countering the increased exposure to MG and thereby countering the potential for increased formation of atherogenic MG-modified LDL [51;57]. There is evidence of a progressive decline of Glo1 expression and activity in ageing – reviewed in [9]- and Glo1 inducers may counter this. Dietary bioactive inducers of Glo1 may provide for resistance to development of ageing related disorders or healthy ageing.

Investigation of species conservation of ARE1 in the *GLO1* gene showed high sequence identity in primates and high sequence identity and similarity in mouse, rat, pig and

cow, and with the overall consensus human ARE motif for basal and inducible expression [58] (Figure 4B). This regulation is conserved in mammalian cells and likely extends to other species with a functional Nrf2 system and orthologues – such as SKN-1 of nematodes; increased Glo1 expression was found in experimental oxidative stress in the parasitic nematode *Onchocerca volvulus* [59]. This further underlines the importance of regulatory increase of cell defences against dicarbonyl stress through the Nrf2/ARE/Glo1 interaction revealed herein.

AUTHOR CONTRIBUTION

M.X, N.R. and P.J.T. designed the study, M.X, N.R., H.M., P.I., M.M.A. and P.J.T. performed experiments and analysed the data, T.S. collected mouse urine samples overseen by T.T. and M.Y., N.K. and B.K.P established Nrf2(-/-) mouse colony and collected mouse liver samples, and PJT wrote the paper with contributions from all authors. The authors declare no competing financial interests.

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FIGURE LEGENDS

Figure 1. Dicarbonyl stress-associated damage to protein and DNA by methylglyoxal, the glyoxalase system and *GLO1* as an ARE-linked gene. (A) Formation of hydroimidazolone MG-H1 residues in proteins. (B) Formation of imidazopurinone MGdG residues in DNA. (C) The glyoxalase system. (D) Reporter response for empty vector, quinone reductase ARE (ARE-NQO1; positive control), and putative *GLO1* ARE serial deletion fragments, ARE-1, ARE-1,2 and ARE-1,2,3. (E) Reporter response for empty vector, NQO-ARE, *GLO1*-ARE1 mutant (ARE-1m) and *GLO1*-ARE full length with ARE-1 mutant (ARE-1m,2,3). Nrf2 activator: SFN (4 μ M). (F) Agarose gel with ethidium bromide staining for control (Con)- and SFN-treated total cell DNA (input), non-immune IgG precipitated DNA controls and anti-Nrf2 IgG precipitated DNA test samples. (G) Relative amount of *GLO1* ARE-1 in test samples. (H) Effect of hydrogen peroxide induced Nrf2/ARE transcriptional response for NQO1 and *GLO1*. Key: open bars – control; solid bars + 500 μ M hydrogen peroxide. Cells were incubated for 6 h after treatment. Data in panels D, E, G and H are mean \pm SD (n = 3). Data in panels D and E are normalised to percentage of ARE-NQO1 positive control, data in panels G normalised to unstipulated control, and data in panel H normalised to unstimulated NQO1-ARE vector transfected control. Significance: *, P<0.05 **, P<0.01 and ***, P<0.001 (*t*-test) – with respect to empty vector control (panels D and E), unstimulated control (panels G and H).

Figure 2. Induction of glyoxalase 1 expression by sulforaphane and allyl isothiocyanate in human HepG2 cells and BJ fibroblasts *in vitro*. Induction of Glo1 activity in HepG2 cells *in vitro*: (A) 2 μ M SFN and (B) 2 μ M AITC. HepG2 cells were incubated with inducer for 24 h. Induction of Glo1 expression in BJ fibroblasts *in vitro* by SFN and protection against protein damage: Glo1 mRNA concentration-response and time course, (C) SFN concentration-response profile incubated for 16 h, time course with 2 μ M SFN – (D) Glo1 mRNA and (E) Glo1 protein, (F) effect of Nrf2 siRNA on Glo1 mRNA with and without 2 μ M SFN; MG concentration in BJ fibroblast cultures – (G) culture medium and (H) BJ cells incubated with 2 μ M SFN for 24 h; and (I) MG-H1 content of cell protein of BJ cells incubated with 2 μ M SFN for 48 h. ChIP assay for *GLO1* ARE-1. Data are mean \pm SD (n = 3 for all panels). Significance: *, P<0.05; **, P<0.01; and ***, P<0.001 (*t*-test).

Figure 3. Increased glyoxalase 1 expression through the Nrf2 system decreases mutation and cell detachment, and translates to regulation of Glo1 expression and prevention of protein and DNA damage *in vivo*. (A) Prevention of frameshift mutations in HepG2 cells *in vitro* induced by 400 μ M MG (400MG) by 1 μ M AITC. (B) Improved HMEC-1 endothelial cell adhesion to type IV collagen conditions with medium from BJ cells incubated without (control) and with 2 μ M SFN for 24 h. Data are mean \pm SD (n = 3). (C) – (I) Expression of Glo1 in wild type Nrf2(+/+) and Nrf2(-/-) mice. (C) Liver Glo1 mRNA, (D) – (H) Glo1 protein liver, brain, heart, kidney and lung. (I) Glo1 protein in wild type Nrf2(+/+) and Nrf2(-/-) mice with and without treatment with CDDO-Me. (J) and (K) Urinary excretion of MGdG and MG-H1 free adducts, respectively, of wild-type Nrf2 (+/+) and Nrf2 (-/-) mice. Data are: median (lower – upper quartile) for C (Nrf2(+/+), n = 5; (Nrf2(-/-), n = 6), D (Nrf2(+/+) and (Nrf2(-/-), n = 6), J and K (Nrf2(+/+), n = 8 and (Nrf2(-/-), n = 9); and mean \pm SD for E (Nrf2(+/+) and (Nrf2(-/-), n = 4), F and G (Nrf2(+/+) and (Nrf2(-/-), n = 6), H (Nrf2(+/+), n = 5 and (Nrf2(-/-), n = 6), and I (Nrf2(+/+) and (Nrf2(-/-), n = 6). Significance: *, P<0.05, **, P<0.01 and ***, P<0.001; *t*-test for parametric data (A, B and E – I) and *Mann-Whitney U* test for non-parametric data (C, D, J and K).

Figure 4. Transcriptional control of *GLO1* by Nrf2 for enhanced protection of the proteins and DNA from dicarbonyl glycation damage. (A) Schematic summary, and (B) Conservation of ARE-1 in exon-1 of the *GLO1*. *Consensus ARE is a weighted consensus ARE derived from comparisons of human, mouse and rat ARE-linked genes [58]. Grey filled entries indicate disparity with human *Glo1* ARE of exon-1.

Table 1. *GLO1* promoter cloning primers.

Deletion mutant	Forward	Reverse	ARE-like sequence:	Size of fragment
F1	<u>TTGGT</u> ACCTGCCCAACCTCATTTTGGTTA	<u>TTGCTAG</u> CTGGCTGAACTGCAGTATCACA	ARE-1,2,3	1176
F2	<u>TTGGT</u> ACCTCACTTCAGCCCAGGAGT	<u>TTGCTAG</u> CTGGCTGAACTGCAGTATCACA	ARE-1,2	850
F3	<u>TTGGT</u> ACCTGCCTCCTTTATGCGCAAG	<u>TTGCTAG</u> CTGGCTGAACTGCAGTATCACA	ARE-1	256
F1	<u>TTGGT</u> ACCTGCCCAACCTCATTTTGGTTA	<u>TTGCTAG</u> CACTGACCCTCTCTGAGCTTCC	ARE-3	622
ARE1 mutant		<u>TTGCTAG</u> CTGGCTGAACTGCAGTATCACA	ARE-1m,2,3	
F1	<u>TTGGT</u> ACCTGCCCAACCTCATTTTGGTTA	<u>TTGCTAG</u> CTGGCTGAACTGCAGTTATCCAGACGA	ARE-1m,2	1176
F3	<u>TTGGT</u> ACCTGCCTCCTTTATGCGCAAG	<u>TTGCTAG</u> CTGGCTGAACTGCAGTTATCCAGACGA	ARE-1m	256

Note: Upper case letters are mutant bases. The sequences for Kpn1 and Nhe1 restriction sites are underlined.

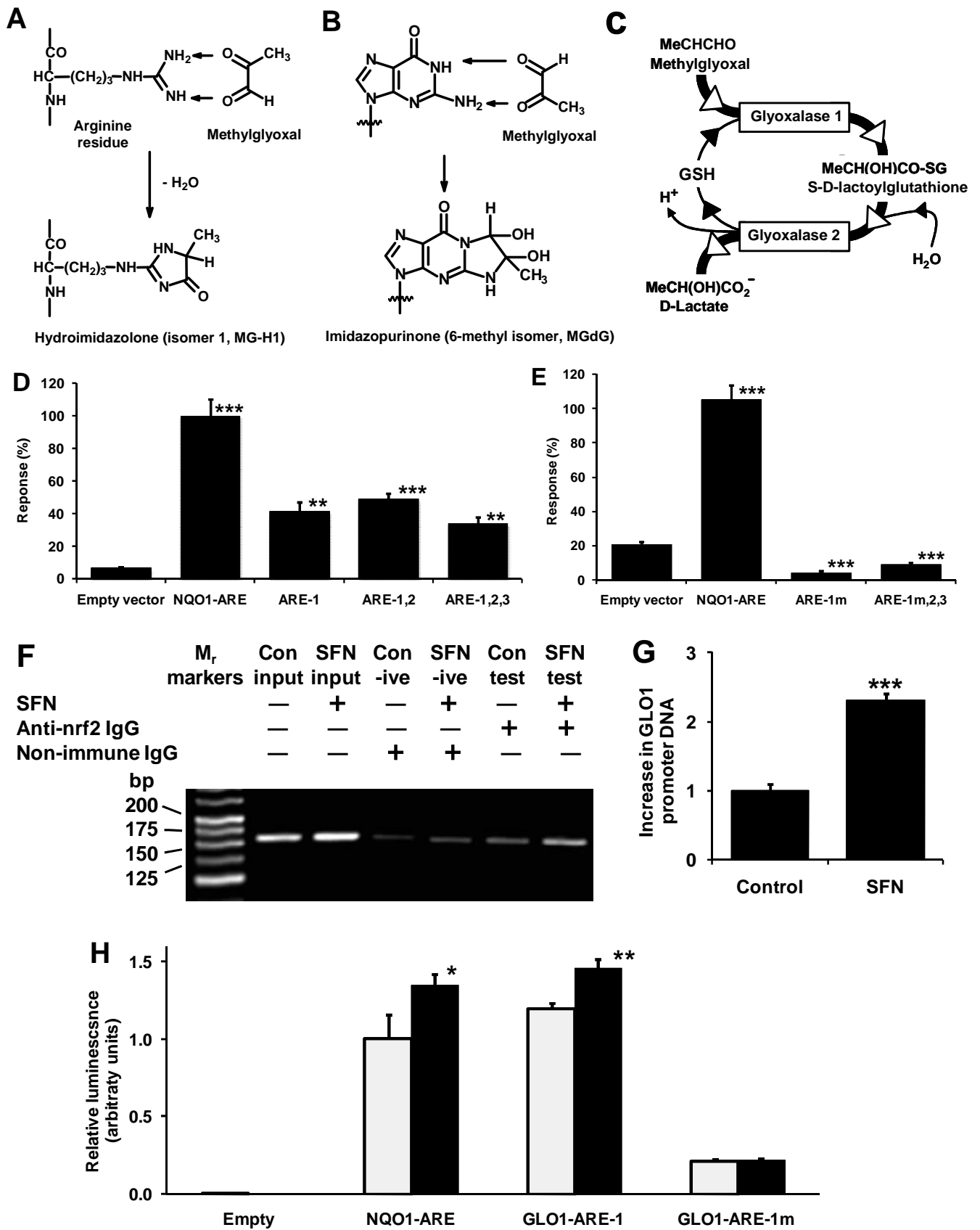


Figure 1 Xue *et al.*, Transcriptional control of glyoxalase 1 by Nrf2

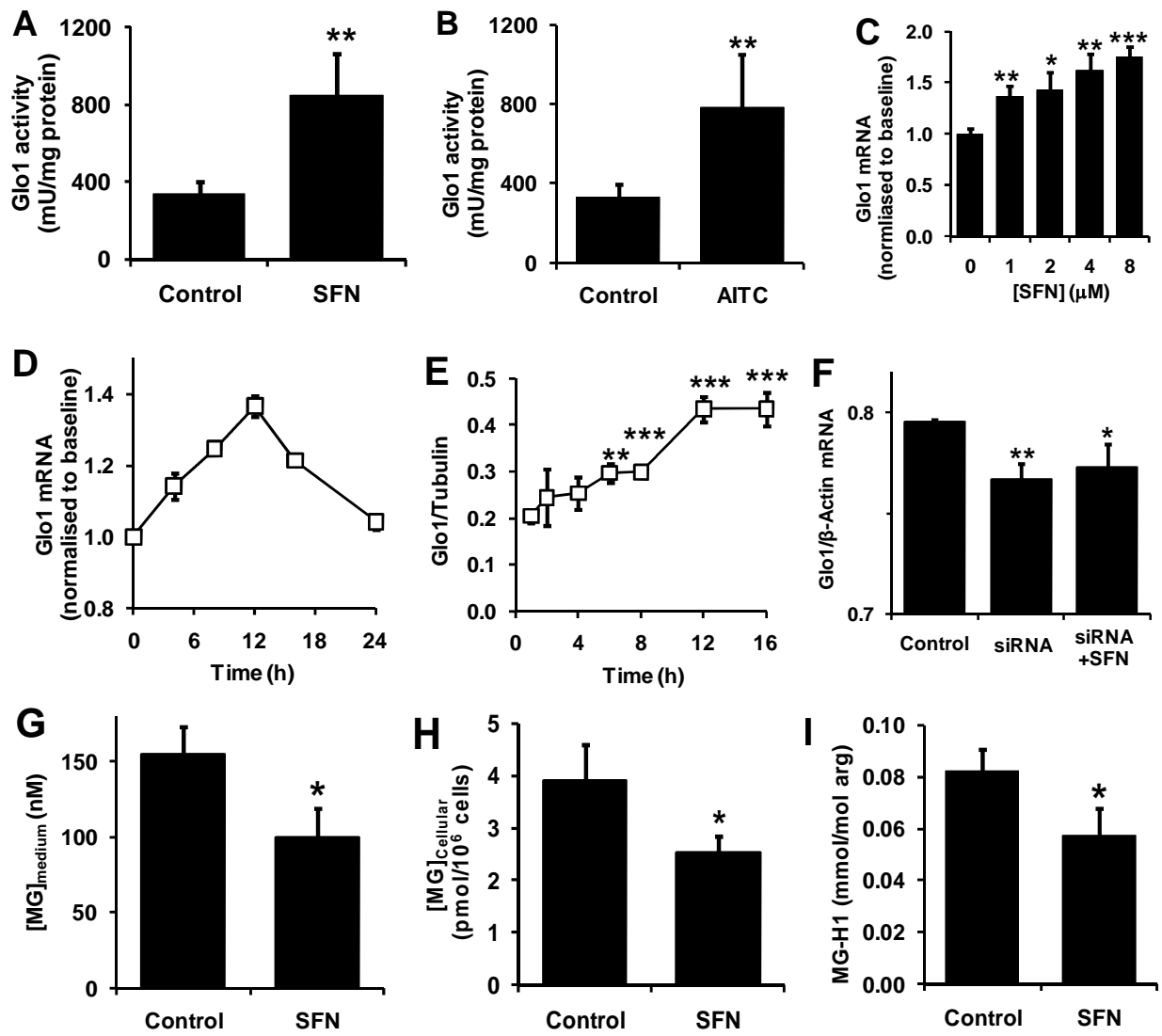


Figure 2 Xue *et al.*, Transcriptional control of glyoxalase 1 by Nrf2

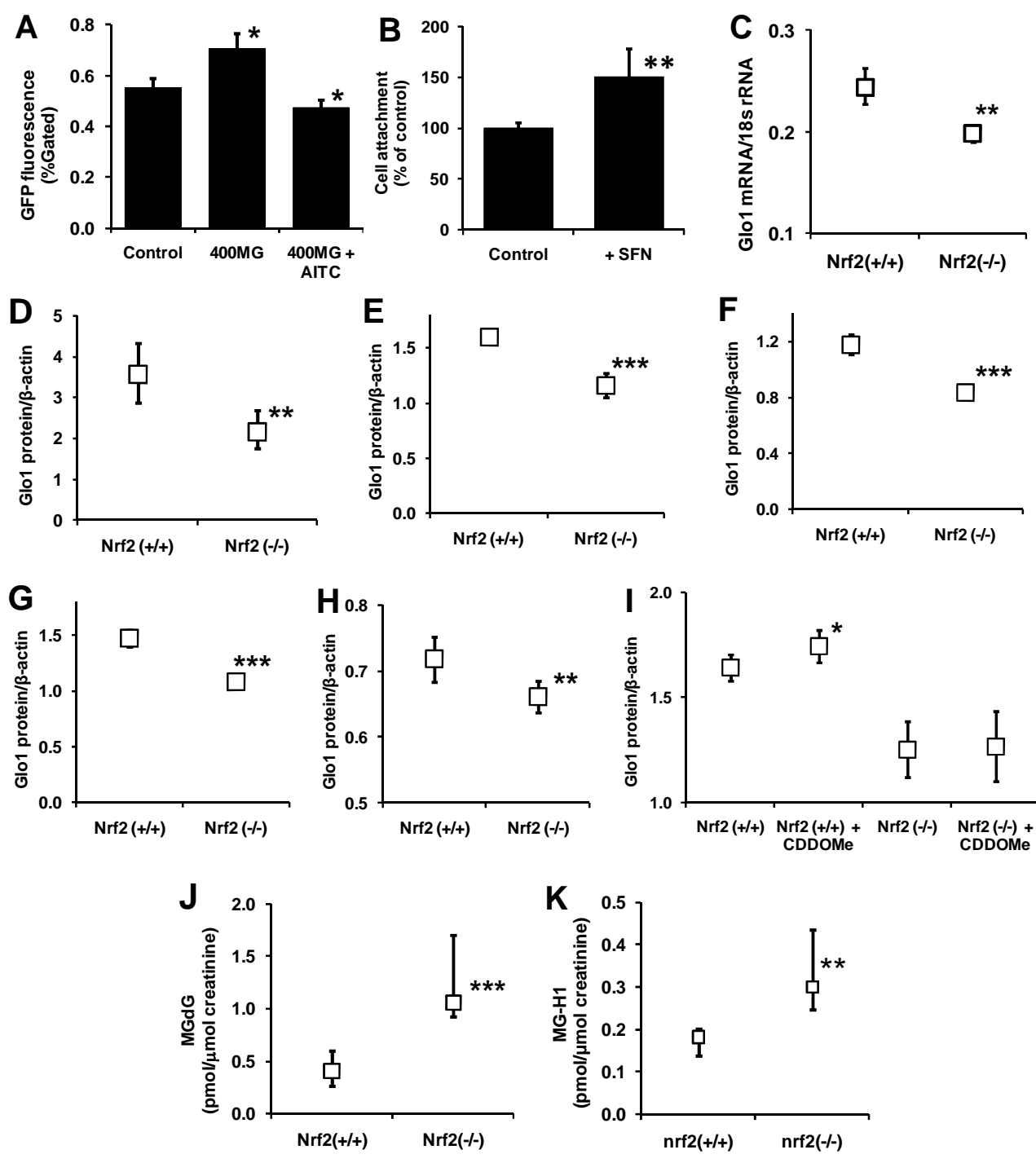
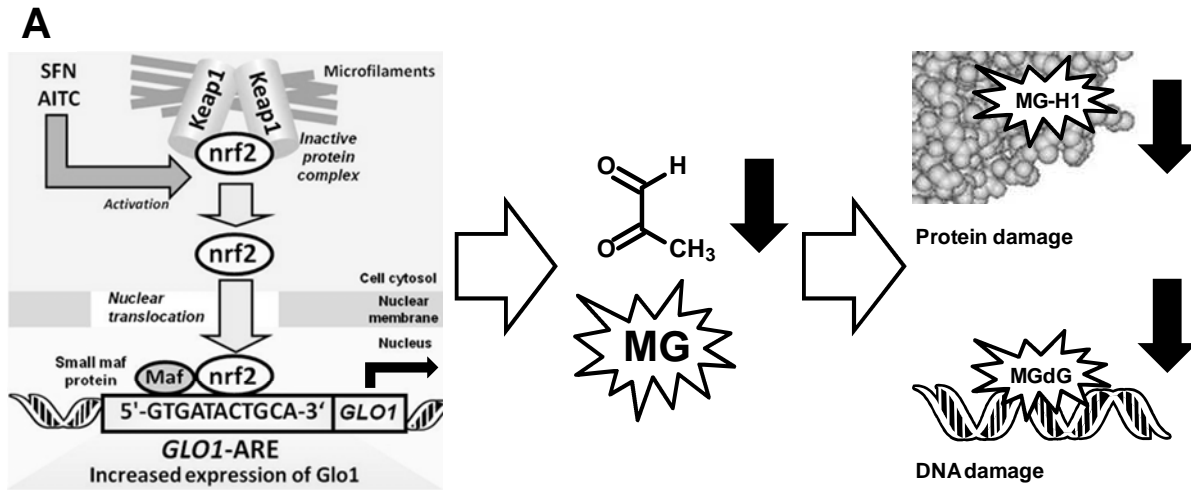


Figure 3 Xue *et al.*, Transcriptional control of glyoxalase 1 by Nrf2



B

Species	ARE1										
Primates											
Human	G	T	G	A	T	A	C	T	G	C	A
Consensus ARE*	G	T	G	A	C	T	C	A	G	C	A
Chimpanzee	G	T	G	A	T	A	C	T	G	C	A
Gorilla	G	T	G	A	T	A	C	T	G	C	A
Macaque	G	T	G	A	T	A	C	T	G	C	A
Marmoset	G	T	G	A	T	A	C	T	G	C	A
Rodents											
Mouse	G	T	G	A	T	T	C	T	C	C	A
Rat	G	C	G	A	T	T	C	T	C	C	A
Rabbit	-	-	-	-	-	-	-	-	G	C	T
Other											
Cow	G	T	G	A	T	A	C	A	G	C	A
Pig	G	T	G	A	T	A	C	-	G	C	A

Figure 4 Xue *et al.*, Transcriptional control of glyoxalase 1 by Nrf2

Transcriptional control of glyoxalase 1 by Nrf2 provides a stress responsive defence against dicarbonyl glycation

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SUPPLEMENTARY DATA

Effect of sulforaphane and allyl isothiocyanate in growth of HepG2 cells and BJ fibroblasts *in vitro*

EXPERIMENTAL

Materials

R-Sulforaphane (SFN) was from LKT Laboratories (St Paul, USA) and allyl isothiocyanate (AITC) and Trypan blue were from Sigma (Poole, UK). Human hepatoma HepG2 cells were obtained from the European collection of Animal Cell Culture (Porton Down, U.K). BJ fibroblasts (human foreskin), originated in the laboratory of J. R. Smith (Baylor College of Medicine, Houston, Texas, USA), and obtained from American Type Culture Collection (ATCC) via LGC Standards (Teddington, Middlesex, UK) at a population doubling of 22.

Cell culture and treatments

Human hepatoma HepG2 cells and human BJ fibroblasts were cultured in MEM medium with 10% fetal calf serum and 2 mM glutamine under an atmosphere of 5% CO₂ in air, 100% humidity and 37°C. Cells were then seeded at 2 - 3 x 10⁴ cells/cm². All cells were manipulated under aseptic conditions. Cell viability was determined by the ability of cells to exclude Trypan blue. The viability of HepG2 cells used in experiments was > 99%.

For study of the effect of SFN and AITC on cell growth and variability, HepG2 cells were cultured at a density 1.2 x 10⁴ cells/cm² and incubated for 48 h at 37°C, 5% CO₂ in air and 100% humidity, with and without 1 – 50 µM test compound. After trypsinization, viable cell and non-viable cell number were determined by the Trypan blue exclusion method [1].

For study of the effect of SFN on growth and variability of BJ fibroblasts, BJ cells were cultured at a density 3 x 10³ cells/cm² and incubated for 48 h at 37°C, 5% CO₂ in air and 100% humidity, with and without 0.5 – 10 µM SFN and, after trypsinization, viable cell number determined.

Statistical analysis of data

Median growth inhibitory concentration GC₅₀ values of SFN and AITC were determined by non-linear regression of viable cell number V (% of untreated control) on compound concentration for the logistic regression equation $V (\%) = GC_{50}^n / (GC_{50}^n + [Compound]^n)$ where n is the logistic regression coefficient, solving for GC₅₀ and n [2]. Non-linear regression was performed with the ENZFITTER programme (Biosoft, Cambridge, UK).

RESULTS

Effect of sulforaphane and allyl isothiocyanate on growth of HepG2 cells and BJ fibroblasts *in vitro*

When HepG2 cells were incubated with SFN and AITC (1 – 50 µM) for 48 h there was a concentration dependent inhibition of growth – Figure S1, A and B. No further growth

inhibition was found beyond this time. Non-linear regression of cell growth data on compound concentration gave: for SFN, $GC_{50} = 13.6 \pm 0.6 \mu\text{M}$ and $n = 1.09 \pm 0.05$ ($N = 17$); and for AITC, $GC_{50} = 22.5 \pm 2.0 \mu\text{M}$ and $n = 1.44 \pm 0.18$ ($N = 24$). When BJ cells were incubated with SFN ($0.5 - 10 \mu\text{M}$) for 48 h there was a concentration dependent inhibition of growth – Figure S1, C. Non-linear regression of cell growth data on SFN concentration gave $GC_{50} = 6.09 \pm 0.31 \mu\text{M}$ and $n = 3.04 \pm 0.05$ ($N = 17$). For concentrations of SFN and AITC employed to study the induction of Glo1 expression and related metabolism ($2 \mu\text{M}$), there was no significant inhibition of growth or toxicity of these isothiocyanates to HepG2 cells or BJ cells.

FIGURE LEGEND

Figure S1. Effect of sulforaphane and allyl isothiocyanate on growth of HepG2 cells and BJ fibroblasts *in vitro* (A) and (B) Effect of SFN and AITC on the growth of HepG2 cells in vitro, respectively. (C) Effect of SFN on the growth of BJ fibroblasts in vitro. Data are mean \pm SD ($N = 3 - 5$). Total data points for the regression equation shown are in the text. Concentration-response curves are typical of outcomes on 3 or more occasions.

REFERENCES

- 1 Kaltenbach, J. P., Kaltenbach, M. H., and Lyons, W. B. (1958) Nigrosin as a dye for differentiating live and dead ascites cells. *Exp.Cell Res.* **15**, 112-117
- 2 Kang, Y., Edwards, L. G., and Thornalley, P. J. (1996) Effect of methylglyoxal on human leukaemia 60 cell growth: modification of DNA, G_1 growth arrest and induction of apoptosis. *Leuk.Res.* **20**, 397-405

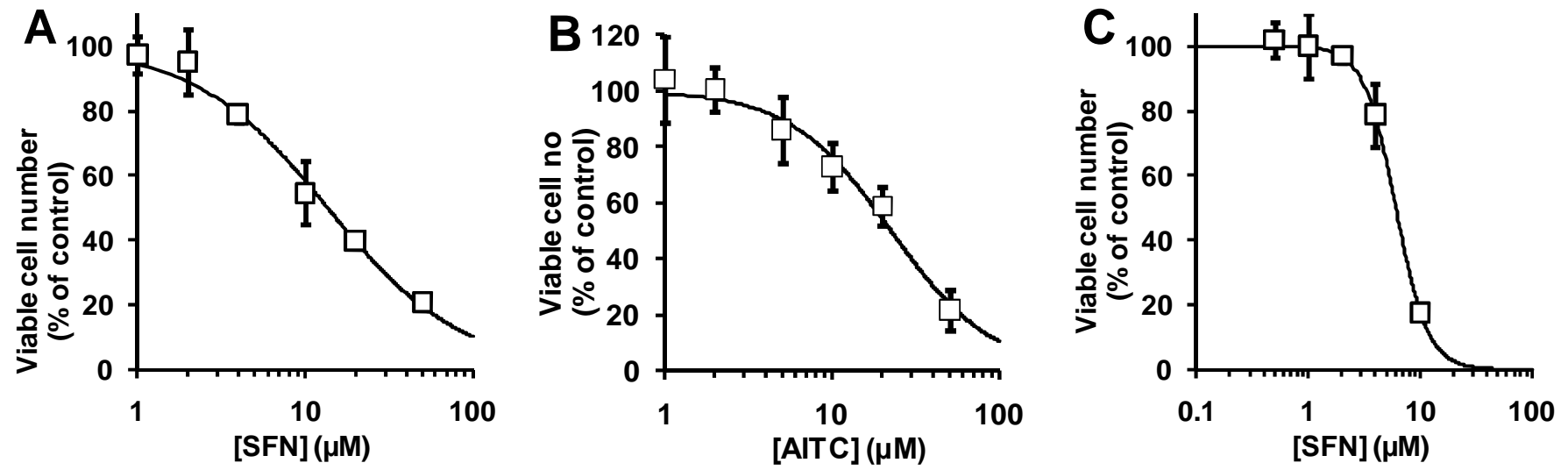


Figure S1 Xue *et al.*, Transcriptional control of glyoxalase 1 by Nrf2