Cellular mechanisms of redox cell signalling: role of cysteine modification in controlling antioxidant defences in response to electrophilic lipid oxidation products

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The molecular mechanisms through which oxidized lipids and their electrophilic decomposition products mediate redox cell signalling is not well understood and may involve direct modification of signal-transduction proteins or the secondary production of reactive oxygen or nitrogen species in the cell. Critical in the adaptation of cells to oxidative stress, including exposure to subtoxic concentrations of oxidized lipids, is the transcriptional regulation of antioxidant enzymes, many of which are controlled by antioxidant-responsive elements (AREs), also known as electrophile-responsive elements. The central regulator of the ARE response is the transcription factor Nrf2 (NF-E2-related factor 2), which on stimulation dissociates from its cytoplasmic inhibitor Keap1, translocates to the nucleus and transactivates ARE-dependent genes. We hypothesized that electrophilic lipids are capable of activating ARE through thiol modification of Keap1 and we have tested this concept in an intact cell system using induction of glutathione synthesis by the cyclopentenone prostaglandin, 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2. On exposure to 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2, the dissociation of Nrf2 from Keap1 occurred and this was dependent on the modification of thiols in Keap1. This mechanism appears to encompass other electrophilic lipids, since 15-A_{2t}-isoprostane and the lipid aldehyde 4-hydroxynonenal were also shown to modify Keap1 and activate ARE. We propose that activation of ARE through this mechanism will have a major impact on inflammatory situations such as atherosclerosis, in which both enzymic as well as non-enzymic formation of electrophilic lipid oxidation products are increased.

Key words: antioxidant defence, electrophilic lipid oxidation, glutamate–cysteine ligase, redox cell signalling, thiol modification, proteomics.

INTRODUCTION

Redox cell signalling involves the post-translational modification of specific signal-transduction proteins by reactive oxygen and nitrogen species (ROS/RNS). It has long been recognized that the secondary reaction products of ROS/RNS with unsaturated fatty acids can generate a spectrum of lipid oxidation products that exhibit a variety of structural and chemical properties. However, it has been difficult to demonstrate the molecular mechanisms that underlie the biological responses mediated by lipid oxidation products. Among these, the electrophilic cyclopentenones are particularly interesting as they can be derived from both specific enzymic pathways and non-specific lipid peroxidation. A frequently employed model system to study the effects of electrophilic lipids is the J series CyPGs (cyclopentenone prostaglandins) synthesized from arachidonic acid via enzymic conversion by cyclooxygenase and PGD_2 (prostaglandin D_2) synthase [1]. These compounds have structural analogues derived from non-enzymic lipid peroxidation [2,3] and are found at the late stages of inflammatory processes [4]. It has been proposed that cyPGs may participate in the resolution of inflammation through inhibition of NF-κB (nuclear factor-κB) activity [5–7]. In human atherosclerotic lesions, immunoreactivity against cyclooxygenase-2, PGD synthase and 15d-PGJ_2 (15-deoxy-$\Delta^{12,14}$-prostaglandin J_2) is increased, suggesting a potential role of PGJs in atherosclerosis [8–10]. Recently, we have proposed that, at low concentrations, electrophilic lipids present in oxLDL (oxidized low-density lipoprotein) or specific compounds such as 15d-PGJ_2 can protect endothelial cells from oxidative stress through the induction of intracellular GSH synthesis [11,12]. The PGJs, including 15d-PGJ_2, can also evoke other cytoprotective responses such as the induction of haem oxygenase-1, presumably by transcriptional induction via the oxidative-stress-activated transcription factor Nrf2 (NF-E2-related factor 2) [13,14].

The rate-limiting enzyme of GSH synthesis is GCL (glutamate–cysteine ligase), which consists of catalytic (GCLC) and modifier (GCLM) subunits, both of which being required for the synthesis of GSH under physiological conditions [15,16]. The subunits are coded for by different genes, and are induced by a variety of oxidative and electrophilic insults [17]. The promoters of both subunits contain antioxidant-responsive elements (AREs, also

Abbreviations used: AP-1, activator protein 1; ARE, antioxidant-responsive element; 15-A_{2t}-isoP, 15-A_{2t}-isoprostane; BIAM, biotin-conjugated iodoacetamide; cyPG, cyclopentenone prostaglandin; PGJ, prostaglandin J; 15d-PGJ_2, 15-deoxy-$\Delta^{12,14}$-PGJ_2; ERK, extracellular-signal-regulated kinase; FBS, foetal bovine serum; GCL, glutamate–cysteine ligase; GCLC and GCLM, GCL catalytic and modifier subunits respectively; GFP, green fluorescent protein; 4-HNE, 4-hydroxy-2-nonenal; HUVEC, human umbilical-vein endothelial cell; IEF, isoelectric focusing; IP, immunoprecipitation; MAPK, mitogen-activated protein kinase; NF-κB, nuclear factor-κB; NQO1, NAD(P)H quinone oxidoreductase-1; hNQO1, human NQO1; oxLDL, oxidized low-density lipoprotein; Nrf2, NF-E2-related factor 2; PI3K, phosphoinositide 3-kinase; PKC, protein kinase C; wt, wild-type.

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referred to as electrophile-responsive elements), with the *cis*-acting sequence being responsible for Nrf2-dependent induction of genes [17–19]. In addition, the GCLC promoter also has an AP-1 (activator protein 1) site that has been reported to mediate the induction by hydrogen peroxide and menadione [20], as well as an NF-κB site, which mediates the induction resulting from exposure to ionizing radiation [21].

ARE was first discovered from the promoter regions of the rat glutathione S-transferase Ya [22] and NQO1 [NAD(P)H quinone oxidoreductase-1] [23] genes; ARE was so named based on the fact that the element is responsive to phenolic antioxidants. It was soon discovered that ARE is responsive to a variety of pro-oxidants and electrophiles, and it regulates a number of other genes important for xenobiotic metabolism and antioxidant defence (see [24] for a review). The consensus ARE sequence, initially characterized by Rushmore et al. [25], was subsequently revised and extended by Wasserman and Fahl [26]. Subsequently, this consensus ARE, containing the sequence 5′-TMAnn**RTGAYnnnGC**Rwwww-3′ (the 'core' ARE sequence is in boldface), was re-assessed by Erickson et al. [27] and Nioi et al. [28], adding variability to the consensus sequence. The GCLC gene has a total of four putative ARE sites, of which one residing approx. 3.1 kb upstream of the transcriptional start site (ARE4) was proven to be functional [19]. The GCLM promoter was initially thought to contain an ARE site at − 302:− 283 and an upstream AP-1 site [18]. This notion was recently revised by Erickson et al. [27], and a second variant ARE site at − 337:− 318, rather than an AP-1 site, was found. This variant ARE has the 'core' ARE sequence 5′-GT**T**ACgaaGC-3′ instead of 5′-RT**G**AYnnnGC-3′ (the variant nucleotide is in boldface and underlined). This nucleotide had previously been considered indispensable [26]. Also, Nioi et al. [28], after examination of the mouse NQO1 promoter, concluded that this guanine within the 'core' ARE sequence makes a less significant contribution to the enhancer activity than considered previously.

The mechanisms by which oxidative stress or electrophile stress evokes an ARE response has been studied extensively. On stimulation, Nrf2 dissociates from its cytoplasmic negative regulator Keap1 and translocates to the nucleus, where it forms dimers with basic region leucine zipper (bZIP) proteins and binds to the ARE sequence [24]. The Nrf2 protein also accumulates in the nucleus through inhibition of its degradation via the 26 S proteasome [29–33]. The inhibition of the ARE response by Keap1 was at first thought to occur merely through the control of the subcellular localization of Nrf2 via direct interaction of the two proteins [34]. However, recent studies by McMahon et al. [30] and Itoh et al. [29] show that, under unstimulated conditions, Keap1 also enhances proteasomal degradation of Nrf2 through its direct interaction with the N-terminal Neh2 domain within Nrf2. This redox-sensitive interaction is disrupted on stimulus, allowing nuclear accumulation of the Nrf2 protein.

Keap1 is a cysteine-rich protein, which makes it an attractive candidate for the sensor of oxidative and electrophilic stress [29]. Recent *in vitro* studies by Dinkova-Kostova et al. [35] using recombinant Keap1 support this concept. Mouse Keap1 has a total of 25 cysteine residues, of which four (Cys^{257}, Cys^{273}, Cys^{288} and Cys^{297}) are sensitive to alkylation. Exposure to electrophiles dose-dependently disrupted the interaction of Keap1 and the Neh2 domain of Nrf2 *in vitro*, suggesting that Keap1 thiols directly sense the electrophile stress [35]. In the present study, we examine the regulation of GSH synthesis by electrophilic lipid oxidation products, and show that the transcriptional induction of the GCL subunits occurs through ARE, involving a mechanism in which covalent modification of cysteine residues in Keap1 plays a critical role.

EXPERIMENTAL

Plasmids

Cloning of the full-length GCLC promoter/reporter transgene (− 3802/GCLC5′-*luc*) and construction of the following have been described previously [18,19]: a deletion mutant lacking the distal ARE in the GCLC promoter (− 2752/GCLC5′-luc), a GCLM promoter (− 1927/GCLM5′-luc), a deletion construct lacking the two tandem AREs in the GCLM promoter (− 1927/GCLM5′Δ − 348: − 183) and the point mutations disrupting the proximal ARE and/or the embedded AP-1 element (m1–m3). The point mutations of the distal ARE in the GCLM promoter (M4 and M5, Figure 2A) or the double ARE mutants were created using the Stratagene XL site-directed mutagenesis kit, using − 1927/GCLM5′-*luc* or the GCLM promoter/luciferase transgene harbouring a mutation in the proximal ARE (m2) [18] as the template. Cloning of the hNQO1 (human NQO1) ARE into pT81-luciferase/enhancer vector, the Keap1 cDNA into p3xFLAG-CMV-10 (Sigma) and the Nrf2 cDNA into pEGFP-C1 (Clontech, Palo Alto, CA, U.S.A.) have been described previously [27,36]. Mutagenesis of the FLAG–Keap1 expression vector was performed with the Stratagene XL site-directed mutagenesis kit using the following HPLC-purified primers (listed $5' \rightarrow 3'$): C257S ($Cys^{257} \rightarrow S$), GGTCAAATACGACaGCCCGCAGCGGCG; C273S, GCGGGCCGTGCGCaGCCATGCGCTCAC; C288S, CGCAGCTGCAGAAGaGTGAGATCCTGCAG; and C297S, GCCGACGCGCGCaGCAAGGACTACC (the mutated nucleotides are indicated by lower-case letters). The correct mutations were verified by sequencing.

Biotinylation and purification of 15d-PGJ_2 and 15-A_{2t}-isoP (15-A_{2t}-isoprostane)

A biotin moiety was added to 15d-PGJ_2 via a carbodi-imide-mediated condensation reaction with 5-(biotinamido)pentylamine (Pierce, Rockford, IL, U.S.A.). The method used was a modification of two previously reported methods [5,37]. Briefly, the reaction mixture consisted of 0.5 mg of 15d-PGJ_2 (Cayman Chemical, Ann Arbor, MI, U.S.A.), 0.29 mg of EDC [1-ethyl-3-(3-dimethylaminopropyl)carbodi-imide-HCl; Pierce] and 0.5 mg of 5-(biotinamido)pentylamine in 90 % (v/v) acetonitrile. The reaction was incubated for 18 h at room temperature (22 °C) and the product was purified by HPLC using a C-18 Luna column (Phenomenex, Torrance, CA, U.S.A.) with a linear gradient from 10 % acetonitrile, 0.24 % acetic acid to 95 % acetonitrile. After extraction with chloroform, the solvent was evaporated under N_2 and the product reconstituted in 100 % (v/v) ethanol. Biotinylation was confirmed by electrospray ionization MS, and the concentration was assessed by measuring absorbance at 306 nm using a molar absorption coefficient of 12 000 $M^{-1} \cdot cm^{-1}$. The biotinylation of 15-A_{2t}-isoP, synthesized as described in [38] and purified by HPLC, was performed in a similar manner, and the concentration was assessed using a molar absorption coefficient of 12 000 $M^{-1} \cdot cm^{-1}$ at 220 nm.

Cell culture

HUVECs (human umbilical-vein endothelial cells) were cultivated as described previously [11]. HEK-293 cells (human embryonic kidney 293 cells; Microbix, Toronto, ON, Canada) were grown in Ham's F12 medium/Dulbecco's modified Eagle's medium (50:50) with 10 % (v/v) FBS (foetal bovine serum), 100 units/ml penicillin, 100 μg/ml streptomycin and 2 mM glutamine. The experiments were performed in a medium containing 2 % FBS unless noted otherwise.

GSH and reporter assays

Total GSH was measured using the recycling assay described in [39]. For luciferase reporter assays, HUVECs or HEK-293 cells were transfected using a SuperFect transfection reagent (Qiagen, Valencia, CA, U.S.A.) according to the manufacturer's instructions. At 20 h post-transfection, the cells were exposed to inducers for 24 h; the cells were then lysed in reporter lysis buffer (Promega, Madison, WI, U.S.A.) and the luciferase activities were measured using the Luciferase Assay System (Promega). The cells were co-transfected with β-galactosidase expression plasmid (pCMVβ; Invitrogen, Carlsbad, CA, U.S.A.) to normalize for transfection efficiency.

Western-blot analysis of GCL subunits

Total cellular protein (40 μg) was fractionated by denaturing electrophoresis, transferred on to a PVDF membrane and probed for GCLC or GCLM using polyclonal antibodies, detected and quantified by electronic chemiluminescence, as described previously [11].

Modification of Keap1 with biotin-15d-PGJ_2, 15-A_{2t}-isoP or 4-HNE (4-hydroxy-2-none) and detection of free thiol groups in Keap1

To detect the binding of biotin-15d-PGJ_2 or biotin-15-A_{2t}-isoP to Keap1, HEK-293 cells were transfected with p3xFLAG-Keap1 or empty p3xFLAG-CMV-10 vector. At 48 h post-transfection, the cells were treated for 1 h in media without FBS with either 10 μM biotin-15d-PGJ_2 or 20 μM biotin-15-A_{2t}-isoP. Cells were washed with Hanks balanced salt solution, harvested by scraping, and lysed into a buffer containing 50 mM Tris/HCl (pH 7.4), 150 mM NaCl, 1 % Triton X-100 and protease inhibitor cocktail (Roche, Indianapolis, IN, U.S.A.). Cell lysates were precleared using Protein A–Sepharose (Amersham Biosciences, Piscataway, NJ, U.S.A.), and lysates containing 200 μg of protein were incubated with 50 μl of UltraLink Immobilized Neutravidin Plus beads (Pierce) overnight at 4 °C with constant shaking. Beads were washed and incubated with SDS denaturing buffer and then subjected to SDS/PAGE and transferred on to PVDF membranes. FLAG–Keap1 was detected using an HRP (horseradish preoxidase)-conjugated mouse anti-FLAG M2 monoclonal antibody (Sigma). The blots were developed using SuperSignal West Dura extended duration substrate (Pierce) and chemiluminescence was detected using FluorChem Imaging System (Alpha Innotech, San Leandro, CA, U.S.A.). In addition, cell lysates were subjected to immunoprecipitation (IP) using an anti-FLAG M2 antibody overnight at 4 °C, followed by incubation with Protein A–Sepharose for 3 h. The beads were then washed with lysis buffer and the protein was eluted as above. The samples were subjected to electrophoresis, transfer and detection with HRP-conjugated avidin (VectaStain ABC Elite kit; Vector Laboratories, Burlingame, CA, U.S.A.). To detect 4-HNE-modified Keap1, the HEK-293 cells transfected with FLAG-tagged Keap1 were treated with 20 μM 4-HNE (Calbiochem, La Jolla, CA, U.S.A.) for 1 h, followed by anti-FLAG IP as above. The 4-HNE-modified protein was detected using anti-HNE-Michael adduct rabbit antisera (Calbiochem). Analysis of protein lysates by two-dimensional IEF (isoelectric focusing) and SDS/PAGE was performed in duplicate using 200 μg of total cell lysate, with separation in the first dimension on a pH 3–10 gradient, followed by resolution on either 10 or 15 % SDS/polyacrylamide gels. Proteins from one gel were detected by silver staining and proteins from the other gel were transferred on to nitrocellulose, and biotinylated proteins were detected using HRP-conjugated streptavidin (Amersham Biosciences) as described below.

To determine if 15d-PGJ_2 was binding to FLAG-tagged Keap1, HEK-293 cells were treated with biotin-conjugated 15d-PGJ_2 as described in [40]. Protein lysates were then subjected to FLAG-IP or neutravidin pull-down as above.

Immunocytochemistry

HEK-293 cells grown on poly-L-lysine-coated coverslips were transfected with GFP (green fluorescent protein)–Nrf2 and FLAG–Keap1. At 48 h post-transfection, the cells were treated with 10 μM 15d-PGJ_2 for 1 h, after which the cells were washed with PBS and fixed with 2 % (w/v) paraformaldehyde. After permeabilization, coverslips were blocked with 1 % BSA, followed by incubation with the mouse anti-FLAG M2 monoclonal antibody (1:1000 dilution) for 1 h. The secondary antibody was Texas Red® X-conjugated goat anti-mouse antibody (Molecular Probes, Eugene, OR, U.S.A.). Images were obtained using an Olympus X170 microscope and analysed with IP LAB SPECTRUM software (Scanalytics, Fairfax, VA, U.S.A.).

Statistical analyses

Results are presented as means $\pm$ S.E.M. Comparisons were made by Student's t test and $P < 0.05$ was considered statistically significant.

RESULTS

A common chemical property shared by cyPGs is that they contain one or two electrophilic α,β-unsaturated carbonyl moieties (Figure 1). We therefore hypothesized that the induction would be mediated through the AREs in the promoter regions of the GCLC and GCLM genes. To examine this, we used either full-length promoter/reporter transgenes or deletion constructs devoid of the regions containing AREs (Figure 2A). Both full-length GCLC and GCLM luciferase transgenes were induced by 15d-PGJ_2 (Figure 2B). However, in the deletion mutant transgene lacking the distal region of the GCLC (-2752/GCLC5′-*luc*), the basal activity of the transgene was decreased by approx. 70 % and the inducible expression was completely abolished (Figure 2B). Similarly, the GCLM transgene in which the region between -348 and -183 is deleted has only approx. 20 % of the activity of the full-length GCLM transgene under basal conditions and no significant induction on stimulation with 15d-PGJ_2 (Figure 2B). We therefore conclude that the region between -3702 and -2752 of the GCLC promoter and the region between -348 and -183 of the GCLM promoter are required for both basal as well as 15d-PGJ_2-inducible expression of their respective promoter-luciferase transgenes in HUVECs. To confirm that 15d-PGJ_2 is effective in mediating endogenous GCLC and GCLM induction, we measured the content of the GCL subunits in non-transfected HUVECs after 24 h exposure to 5 and 10 μM 15d-PGJ_2 (Figure 2C) and the intracellular content of GSH after 16 h exposure to the same concentrations (Figure 2D). It is clear that both GCLM and GCLC proteins increase in response to 5 and 10 μM 15d-PGJ_2, in a concentration-dependent fashion (Figure 2C). Similarly, intracellular GSH increases with 15d-PGJ_2 treatment (Figure 2D), confirming that the results, obtained using the reporter constructs, reflect endogenous gene activity.

To study the functionality of the two AREs in the GCLM promoter, mutations of the proximal ARE site and/or the AP-1 site embedded within (m1–m3), the distal ARE site (M4 and M5) or both proximal and distal ARE sites (m2M4 and m2M5) were introduced into the -1927/GCLM5′-*luc* transgene using site-directed mutagenesis (Figure 3A). In transgenes in which only

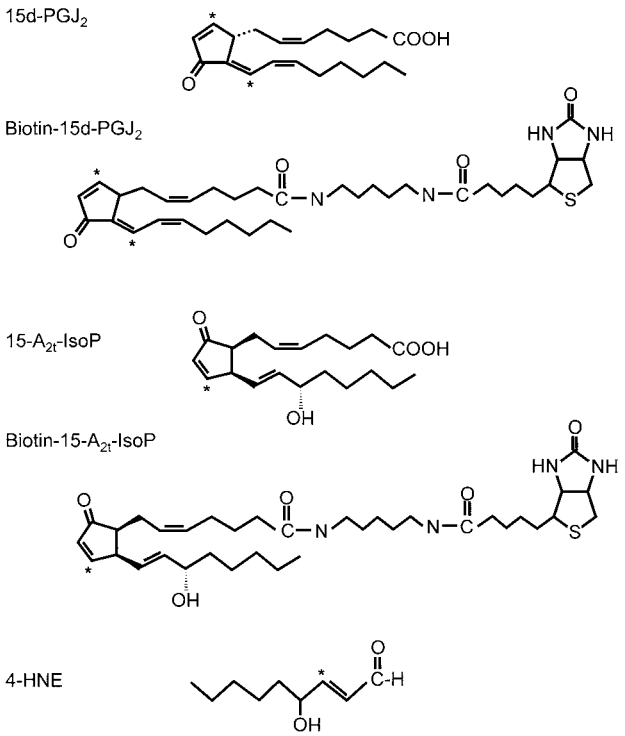

Figure 1 Structures of 15d-PGJ$_2$, biotin-15d-PGJ$_2$, 15-A$_{2t}$-isoP, biotin-15-A$_{2t}$-isoP and 4-HNE

Electrophilic carbons are depicted by asterisks.

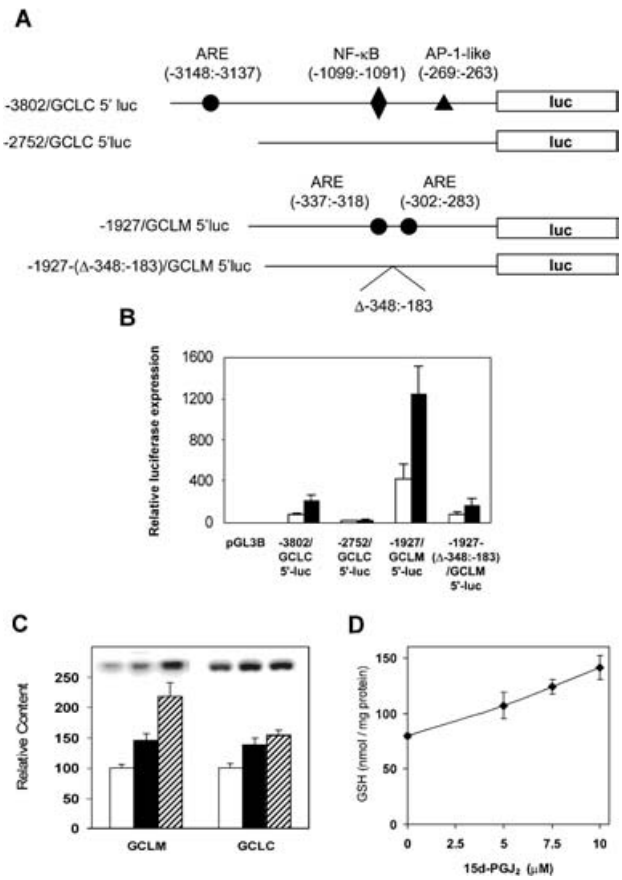

Figure 2 Activation of GCLC and GCLM transgenes and induction of GCLC and GCLM proteins and GSH by 15d-PGJ$_2$

(**A**) A schematic representation of the GCLC and GCLM promoter/reporter transgenes used in the present study. The putative ARE, AP-1 and NF-κB sites are depicted. (**B**) HUVECs were transiently transfected with the promoter/reporter transgenes depicted in (**A**). At 20 h post-transfection, the cells were exposed to vehicle (open bars) or 2.5 μM 15d-PGJ$_2$ (black bars) for 24 h. (**C**) Western-blot example (top) and quantification of blots (bottom) for GCLC and GCLM after exposure to 5 and 10 μM 15d-PGJ$_2$ for 24 h in HUVECs. (**D**) Intracellular content of GSH after 16 h exposure to 5 and 10 μM 15d-PGJ$_2$. Results are the means $\pm$ S.E.M. for three independent measurements.

the proximal ARE and/or the embedded AP-1 site was mutated (m1–m3), or alternatively, only the distal ARE was mutated (M4 and M5), the inducibility of the luciferase transgenes on exposure to 15d-PGJ$_2$ is retained (Figure 3B). The 15d-PGJ$_2$-inducible expression of GCLM luciferase was completely abolished only when both the proximal and distal AREs were mutated (m2M4 and m2M5; Figure 3B).

To test the hypothesis that 15d-PGJ$_2$ would directly react with Keap1, we used FLAG-tagged Keap1 protein and biotinylated 15d-PGJ$_2$. Owing to the greater transfection efficiency, as well as higher levels of protein expression, HEK-293 cells were used for these studies. The biotin tag was introduced at the C-terminus, leaving the two electrophilic β-carbons potentially available for Michael addition reactions (Figure 1). Treatment with 5 μM 15d-PGJ$_2$ for 24 h increased -1927/GCLM5′-*luc* transgene activity in these cells by 2.4 ± 0.2-fold ($P < 0.01$, $n = 3$).

Cells transfected with FLAG-tagged Keap1 were treated with biotin-15d-PGJ$_2$, followed by either affinity purification using neutravidin beads or IP using an antibody raised against the FLAG epitope. In the cell lysates selected for biotin-containing proteins, immunodetection using the anti-FLAG antibody revealed a 66 kDa protein consistent with FLAG–Keap1. This protein was not found in cells that were either mock-transfected or not treated with biotin 15d-PGJ$_2$ (Figure 4A). Conversely, in cells transfected with FLAG–Keap1 and treated with biotin-15d-PGJ$_2$, FLAG-IP recovered a 66 kDa protein that reacted with avidin HRP, consistent with the formation of a covalent adduct with biotin-15d-PGJ$_2$ (Figure 4B). Recovery of the FLAG–Keap1 protein in lysis buffer containing 1 % Triton X-100 was similar in both treated and untreated controls (Figure 4B).

To examine whether the thiol residues in Keap1 were the targets of 15d-PGJ$_2$ modification, free thiol groups were labelled with BIAM (biotin-conjugated iodoacetamide) under acidic conditions. The reactive thiol residues (Cys^{257}, Cys^{273}, Cys^{288} and Cys^{297}) in mKeap1 (mutated Keap1) characterized in the purified recombinant protein are adjacent to basic residues; therefore, they are expected to have lower pK_a values and to be more reactive [35]. When FLAG–Keap1-transfected cells were treated with unmodified 15d-PGJ$_2$, reacted with BIAM and purified using neutravidin beads, the recovery of the FLAG–Keap1 protein was less when compared with that in non-treated controls (Figure 4C). Conversely, when the lysates were immunoprecipitated with an anti-FLAG antibody followed by detection using avidin HRP, the labelling of Keap1 protein thiols was decreased in 15d-PGJ$_2$-treated cells (Figure 4D). Taken together, these results demonstrate that the thiols in Keap1 are the targets of the modification by the electrophilic lipid.

To verify that both biotin-15d-PGJ$_2$ and 15d-PGJ$_2$ can activate ARE in HEK-293 cells, the hNQO1 ARE and a mutation disrupting the core ARE (Figure 4E) were used. Both 15d-PGJ$_2$ and biotin-15d-PGJ$_2$ were capable of causing a significant ($P < 0.05$) increase in the activity of the wt (wild-type) but not the mutated reporter transgene.

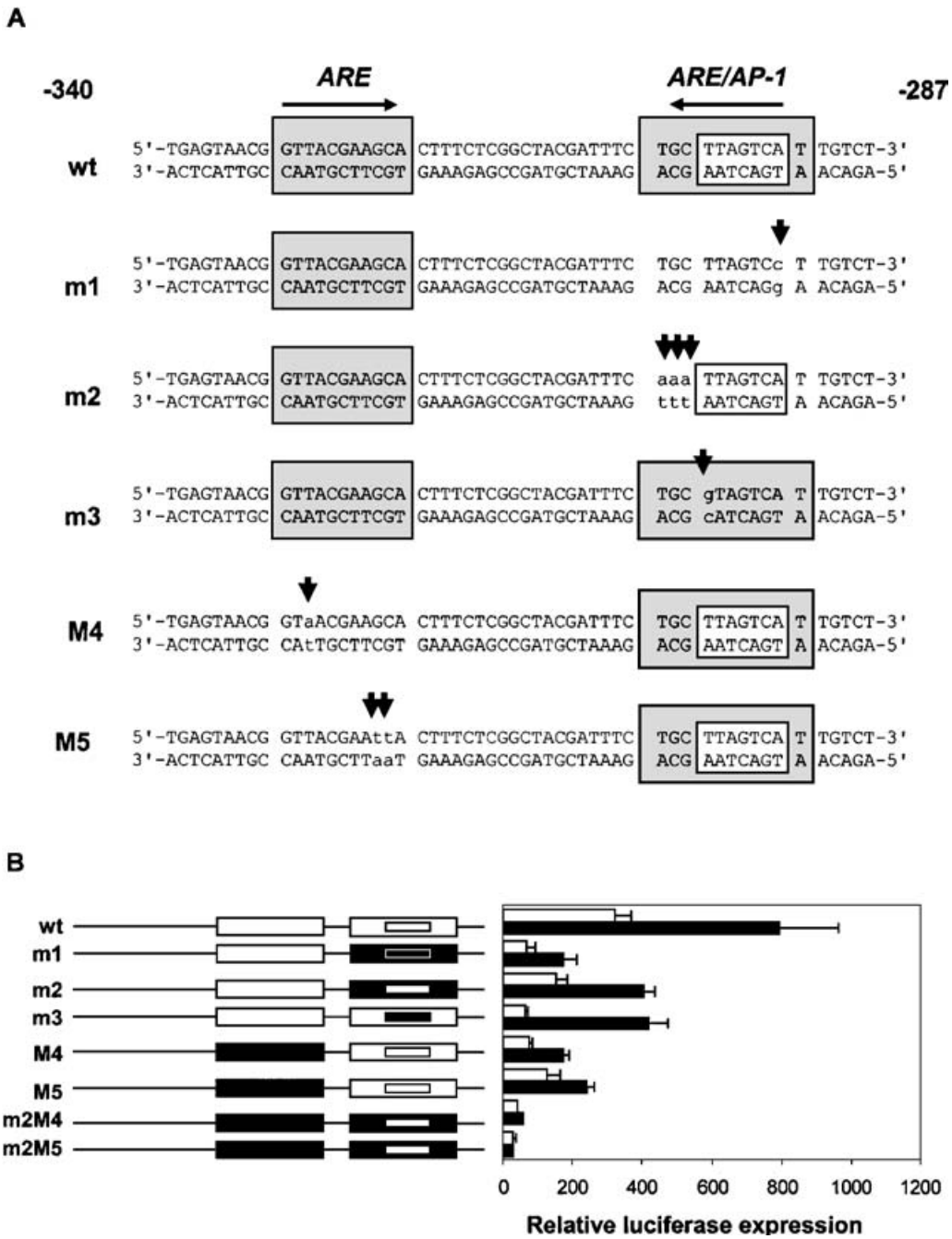

Figure 3 Effect of mutations in the ARE sequences and/or AP-1 on basal and 15d-PGJ$_2$-induced expression of the − 1927/GCLM5′-*luc* transgene

(**A**) The sequence of the region between − 349 and − 287 of wt and mutation constructs harbouring a mutation in the ARE site proximal to the transcription start site (− 302:− 283) and/or in the embedded AP-1 site (m1–m3), or in distal ARE (− 337:− 318). The shaded rectangles depict core AREs and the open rectangle an internal AP-1 site in the proximal ARE sequence. The absence of the rectangle indicates a mutation of the element. Mutated nucleotides are indicated by arrows and lower-case letters. (**B**) The effect of GCLM mutations on basal (right panel, open bars) and 15d-PGJ$_2$-induced (right panel, black bars) expressions was studied as in Figure 2(B). Each transgene is represented by a box in the left panel: open box, wt response element; black box, mutated element. Experimental details are the same as in Figure 2(B). Results are the means ± S.E.M. ($n = 3$).

To determine the effect of 15d-PGJ$_2$ on the subcellular localization of Nrf2 and Keap1, GFP–Nrf2 and FLAG–Keap1 were co-expressed in HEK-293 cells, followed by detection using fluorescence microscopy. In control cells transfected with GFP–Nrf2 and FLAG–Keap1, both proteins were localized in the cytoplasm (Figure 5A). However, after treatment with 10 μM 15d-PGJ$_2$ for 1 h, GFP fluorescence was found almost exclusively in the nucleus, demonstrating that 15d-PGJ$_2$ is capable of liberating Nrf2 from Keap1, enabling its nuclear translocation.

The co-expression of GFP–Nrf2 with the GCLM transgene caused an approx. 9-fold increase in the luciferase activity, demonstrating the function of the regulatory system under these conditions (Figure 5B). This increased activity of the GCLM reporter was repressed by increasing concentrations of FLAG–Keap1 expression plasmid (Figure 5B). However, when the cells were exposed to increasing concentrations of 15d-PGJ$_2$, the ability of Nrf2 to transactivate the GCLM promoter was restored, despite the presence of Keap1 (Figure 5C).

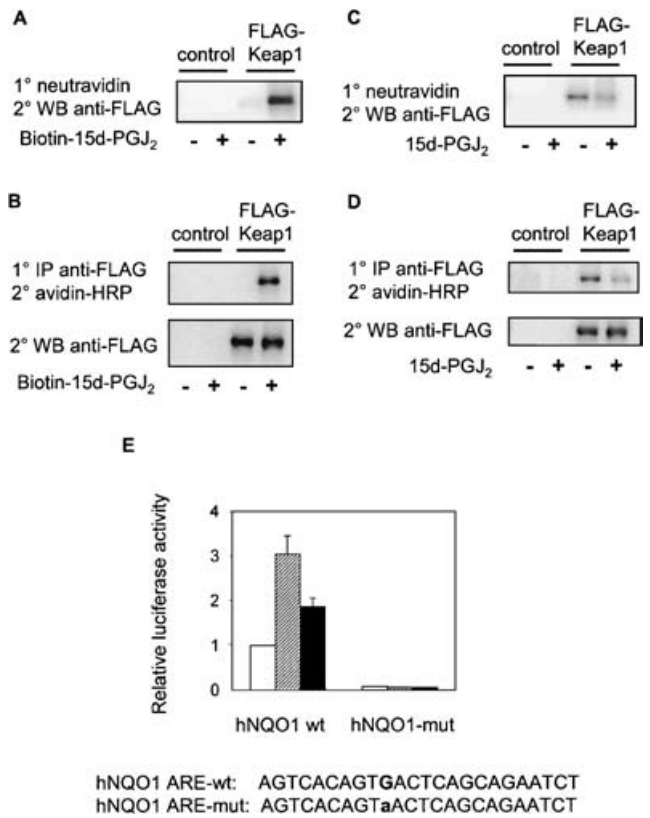

Figure 4 Binding of 15d-PGJ$_2$ to thiols of Keap1

(**A**, **B**) HEK-293 cells transfected with either the empty p3xFLAG-CMV-10 vector or p3xFLAG-Keap1 were treated with 10 μM biotin-15d-PGJ$_2$ for 1 h, followed by neutravidin sequestration of biotin-containing proteins by neutravidin pull-down (**A**) or IP against the FLAG epitope (**B**). Representative blots of six independent experiments are shown. (**C**, **D**) HEK-293 cells transfected with the empty p3xFLAG-CMV-10 vector or p3xFLAG-Keap1 were treated with 10 μM 15d-PGJ$_2$ for 1 h, followed by cell lysis and labelling of thiol residues with BIAM and neutravidin sequestration of biotin-containing proteins (**C**) or FLAG-IP (**D**). (**E**) Activation of hNQO1 ARE in HEK-293 cells by 15d-PGJ$_2$. HEK-293 cells were transfected with either the wt hNQO1 ARE enhancer/reporter transgene or the transgene with a mutation in the core ARE sequence. The cells were then exposed to 5 μM unmodified 15d-PGJ$_2$ (hatched bars) or biotin-15d-PGJ$_2$ (black bars) at 20 h post-transfection, and luciferase and β-galactosidase activities were measured after 24 h. Results are expressed relative to the basal activity of the wt NQO1 ARE, which is depicted to the left. Results are the means $\pm$ S.E.M. ($n = 3$).

To examine the role of the four reactive cysteine residues of Keap1 (Cys^{257}, Cys^{273}, Cys^{288} and Cys^{297}) characterized by Dinkova-Kostova et al. [35] in the activation of GCLM by Nrf2, we mutated these cysteine residues to serine residues individually in FLAG–Keap1 by site-directed mutagenesis, and co-transfected them with Nrf2 (Figure 6). Two of these mutations, C257S and C297S, behaved in a manner similar to that of wt Keap1 (i.e. they were capable of repressing the transactivation of GCLM transgene by Nrf2) (Figure 6A). This repression was reversed after exposure to 15d-PGJ$_2$ in a manner similar to wt Keap1. However, C273S or C288S Keap1 mutations were unable to attenuate the transactivation of GCLM by Nrf2. The wt and mutated proteins were expressed to similar levels in HEK-293 cells, implying that the functional differences between the wt Keap1 and C273S or C288SKeap1 are not due to differences in the translation efficiency or stability of the proteins (Figure 6B).

To assess whether Keap1 could be a target of other electrophilic lipid oxidation products, we selected two structurally distinct compounds, 15-A$_{2t}$-isoP and 4-HNE, which both have a single α,β-unsaturated carbonyl moiety and are formed by non-enzymic lipid peroxidation [2,3,41]. In HEK-293 cells exposed to 20 μM 15-A$_{2t}$-isoP or 4-HNE for 24 h, total GSH was increased by 1.7 ± 0.4- and 1.9 ± 0.1-fold respectively (mean fold increase $\pm$ S.E.M., $n = 3$). However, 15d-PGJ$_2$ was a much more potent inducer of GSH when compared with either 15-A$_{2t}$-isoP or 4-HNE; 2.5 μM 15d-PGJ$_2$ was capable of causing a comparable increase in total cellular GSH levels (2.3 ± 0.1-fold increase compared with control, $n = 3$). Both 15-A$_{2t}$-isoP and 4-HNE at 20 μM could activate the wt, but not the mutated, hNQO1 ARE (Figure 7A).

To determine whether 15-A$_{2t}$-isoP can also react with Keap1, we biotinylated 15-A$_{2t}$-isoP in a manner similar to that of 15d-PGJ$_2$. Using neutravidin pull-down, immunoreactivity against FLAG–Keap1 in cells transfected with FLAG–Keap1 and treated with biotin-15-A$_{2t}$-isoP was evident (Figure 7B). Conversely, incubation with biotin-15-A$_{2t}$-isoP of FLAG–Keap1-transfected cells resulted in an IP recovery of FLAG–Keap1 protein binding avidin HRP (Figure 7C). Similarly, when FLAG–Keap1-transfected cells were treated with 20 μM 4-HNE, immunoreactivity against 4-HNE-Michael adducts was found in the FLAG-IP samples (Figure 7D).

It is anticipated that the reactive electrophilic lipids used in the present study will form covalent adducts with a number of proteins in addition to Keap-1. Other examples include H-Ras, thioredoxin, NF-κB and inhibitory κB kinase [5,7,54,56,57]. To obtain a perspective on the extent of reactivity of proteins in the cell with electrophilic lipids, HEK-293 cells were incubated with a high concentration of biotin-15d-PGJ$_2$ (25 μM) for 1 h and the protein lysates were separated by two-dimensional-IEF and SDS/PAGE. Figure 8(A) shows the results of silver staining of these gels and it was evident, after analysis, that approx. 200 proteins could be detected. A parallel sample was then subjected to Western-blot analysis for biotinylated proteins, and approx. 51 proteins could be detected (Figure 8C). After subtraction for the signal from the endogenous biotinylated proteins (Figure 8B), 29 proteins were identified as reacting with the electrophilic lipid. It is interesting to note that not all of these proteins could be matched with a corresponding spot on the stained gel.

DISCUSSION

In the present study, we examined the mechanism by which 15d-PGJ$_2$ and other electrophilic lipid oxidation products can initiate cytoprotective pathways and we identified Keap1 as the molecular target of electrophilic lipid oxidation products, leading to the release of Nrf2 and its translocation to the nucleus. Our results are consistent with recent *in vitro* studies using the bacterial recombinant Keap1 protein [35]. Recently, Sekhar et al. [42] also showed that exposure to ARE-inducing agents alter the redox state of Keap1 thiols in human Keap1 (KIAA0132). We propose that Keap1 is a rather promiscuous receptor of structurally different electrophilic lipids resulting in an induction of phase II enzymes. This is important at sites of inflammation, such as atherosclerotic lesion, in which lipid oxidation products can be formed through both enzymic as well as non-enzymic pathways [43]. Structural analogues of cyPGs and other electrophilic lipid oxidation products are also found in oxLDL, and these may mediate the effect on ARE-dependent cytoprotective pathways [41,44]. Indeed, oxLDL induces GCL genes in endothelial cells and monocytes through ARE [12,45].

We have demonstrated using site-directed mutagenesis that, among the four reactive cysteine residues characterized by Dinkova-Kostova et al. [35], two (Cys^{273} and Cys^{288}) were critical for negative regulation of the ARE activation by Nrf2 (Figure 6A).

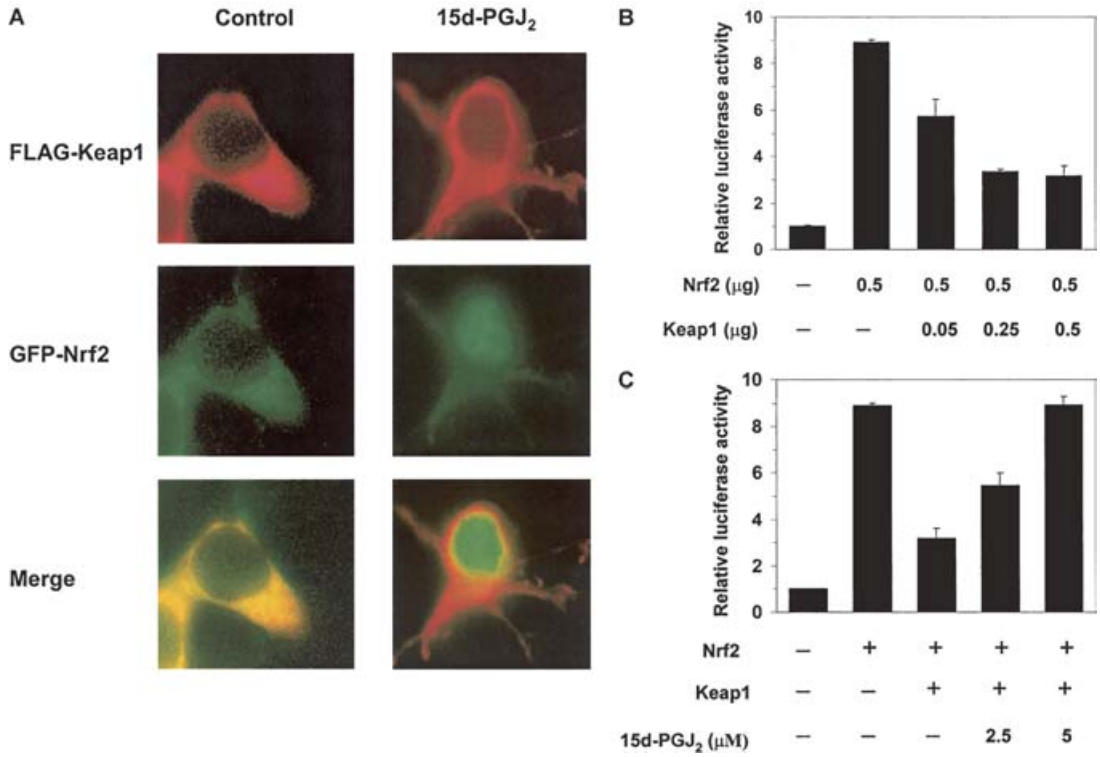

Figure 5 Treatment with 15d-PGJ_2 causes the dissociation of Nrf2 from the repression of Keap1

(**A**) HEK-293 cells grown on glass coverslips were transfected with GFP–Nrf2 and FLAG–Keap1. At 48 h post-transfection, the cells were treated with 10 μM 15d-PGJ_2 for 1 h, after which the cells were fixed and immunostained against the FLAG epitope. (**B**) HEK-293 cells were transfected with 0.25 μg of GCLM-luciferase transgene, 0.5 μg of GFP–Nrf2 and the indicated amounts of p3xFLAG-Keap1. The total amount of DNA was held constant by the addition of empty p3xFLAG-CMV-10 vector. (**C**) HEK-293 cells were transfected with 0.25 μg of GCLM-luciferase transgene, 0.5 μg of GFP–Nrf2 and 0.5 μg of p3xFLAG-Keap1. At 20 h post-transfection, the cells were treated with 15d-PGJ_2 for 24 h. Results in (**B**, **C**) are expressed as fold increase versus basal GCLM promoter activity. Results are the means $\pm$ S.E.M. ($n = 3$).

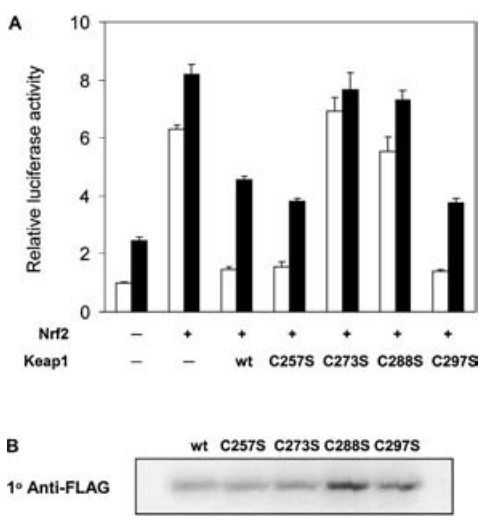

Figure 6 Effect of C257S, C273S, C288S and C297S mutations of Keap1 on the repression of Nrf2-dependent transactivation of GCLM-luciferase transgene

(**A**) HEK-293 cells were transfected with 0.25 μg of GCLM-luciferase transgene, 0.5 μg of GFP–Nrf2 and 0.5 μg of the wt or mutated p3xFLAG-Keap1. At 20 h post-transfection, the cells were treated with vehicle (open bars) or 5 μM 15d-PGJ_2 (black bars). Results are expressed as fold increase versus basal GCLM promoter activity. Results are means $\pm$ S.E.M. ($n = 3$). (**B**) HEK-293 cells were transfected with wt or mutated p3xFLAG-Keap1 expression plasmids. At 48 h post-transfection, the cells were lysed and equal amounts of protein were analysed by Western-blot analysis against the FLAG epitope. The blot shown is representative of three independent experiments.

This is in agreement with the prediction of Zhang et al. [46] based on the phylogenic comparison of the putative reactive cysteine residues. The two cysteine residues, Cys^{273} and Cys^{288}, are conserved in most of the Keap1-related proteins involved in sensing oxidative stress, thereby suggesting that these are probably the critical redox-sensitive cysteine residues for Keap1–Nrf2 interaction. However, mKeap1 has a total of 25 cysteine residues, and nearly all of these can react with a large excess of alkylating agents *in vitro* [35]. A role for the other cysteine residues *in vivo* cannot be ruled out, nor is it possible to exclude at this stage a mechanism in which the C273S and C288S Keap1 mutations could cause conformational changes that disrupt its inhibitory function but are unrelated to the chemical reactivity of thiols. These aspects as well as the exact mechanism by which the Keap1–Nrf2 interaction is disturbed by electrophiles clearly warrant further investigation.

Both 15-A_{2t}-isoP as well as 4-HNE are less potent than 15d-PGJ_2 in activating ARE. This is in accordance with previous results stating that the potency of Michael reaction acceptors to induce ARE depends on their reactivity with thiol groups and correlates with their ability to react with thiols in Keap1 [35,47]. The PGJs have two electrophilic β-carbons, whereas both 4-HNE and 15-A_{2t}-isoP have only one. Moreover, the aldehyde group of 4-HNE is also capable of Schiff base formation with the amino groups of proteins [41], which may compete with the cellular protein targets for the Michael addition reaction. The level of 4-HNE under normal conditions ranges from 0.1 to 3 μM and has been reported to reach 10 μM under conditions of oxidative stress [48]. Under basal conditions, pM to nM concentrations of free 15d-PGJ_2 have been measured from biological fluids, and

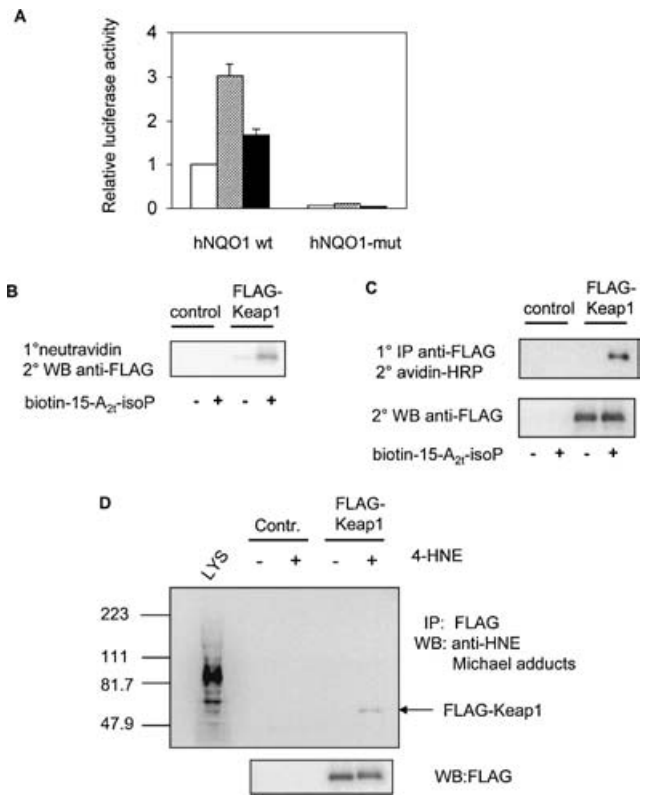

Figure 7 Activation of ARE and modification of Keap1 by other electrophilic lipid oxidation products

(**A**) Activation of hNQO1 ARE in HEK-293 cells by 15-A_{2t}-isoP or 4-HNE. HEK-293 cells were transfected with the wt hNQO1 or mutated ARE as in Figure 4(E). The cells were exposed to 20 μM 15-A_{2t}-isoP (hatched bars) or 4-HNE (black bars), and luciferase activities normalized to β-galactosidase activities were measured and expressed as in Figure 5. Results are means $\pm$ S.E.M. ($n = 3$). (**B**, **C**) HEK-293 cells. transfected with either the empty p3xFLAG-CMV-10 vector or p3xFLAG-Keap1, were treated with 20 μM biotin-15-A_{2t}-isoP for 1 h, followed by neutravidin sequestration of biotin-containing proteins (**B**) or IP against the FLAG epitope (**C**). (**D**) HEK-293 cells, transfected with the empty p3xFLAG-CMV-10 vector or p3xFLAG-Keap1, were treated with 20 μM 4-HNE for 1 h, followed by FLAG-IP and detection of 4-HNE-Michael adducts by Western-blot analysis. LYS, FLAG–Keap1-transfected HNE-treated cell lysate (10 μg) before IP.

increased cyPG levels have been reported during late stages of inflammation [1,4]. A recent report by Bell-Parikh et al. [49], measuring free 15d-PGJ_2, questions the formation of biologically meaningful concentrations of the compound *in vivo*. However, a quantitative estimation of electrophilic lipid oxidation products is complicated by the reactivity of the α,β-unsaturated carbonyl group, which renders them susceptible to conjugation. Electrophilic lipid oxidation products, including 4-HNE, 15-A_{2t}-isoP and PGJs, are effectively conjugated with GSH by glutathione S-transferases [50–52]. It is probable that the measured concentrations of free compounds do not necessarily represent the concentrations to which the cells are exposed locally or the flux of electrophiles with similar reactivity generated by non-enzymic mechanisms of lipid peroxidation.

The fact that electrophilic lipid oxidation products bind to Keap1 protein does not rule out the possibility that they may have an impact on upstream signalling proteins. Multiple protein kinase pathways, such as MAPK (mitogen-activated protein kinase), PI3K (phosphoinositide 3-kinase) and PKC (protein kinase C), have been proposed to play a role in ARE activation, although the role of each pathway in the regulation of ARE and their molecular targets is controversial and probably specific to a given gene and cell type [24]. Many of these kinase pathways seem to be redox-sensitive. For example, protein tyrosine phosphatases have active-site cysteine residues having low pK_a values that are prone to both oxidation and alkylation reactions [53]. Irreversible inactivation of tyrosine phosphatases by electrophiles may then lead to sustained tyrosine phosphorylation and prolonged activation of signalling pathways, such as the MAPK and PI3K/Akt pathways, leading to enhanced activation of ARE-dependent genes. Another recently discovered cell signalling protein directly modified by 15d-PGJ_2 is H-ras, which is activated through modification of Cys^{184} by 15d-PGJ_2 [54]. As *ras* proteins are upstream of both PI3K/Akt and ERK (extracellular-signal-regulated kinase) pathways, modification of these proteins could also have an impact on the activation of ARE-dependent genes. Both these pathways have also been shown to be induced by 15d-PGJ_2 [54]. However, it should be noted that, in human endothelial cells, inhibition of the ERK pathway by PD98059 does not affect the increase in GSH induced by 15d-PGJ_2, arguing against a major contribution from this pathway in mediating the induction of GSH synthesis (results not shown). This is in contrast with results obtained from HepG2 cells, in which ERK1/ERK2 regulates GCLM gene induction by affecting the nuclear translocation of Nrf2 protein [55].

In a proteomic analysis of the proteins forming stable covalent products with the biotin-15d-PGJ_2, it was found that approx. 29 proteins were reactive. This is a relatively small subproteome from the whole cell, in view of electrophilic lipids having the potential to react with other nucleophilic centres, including histidine and

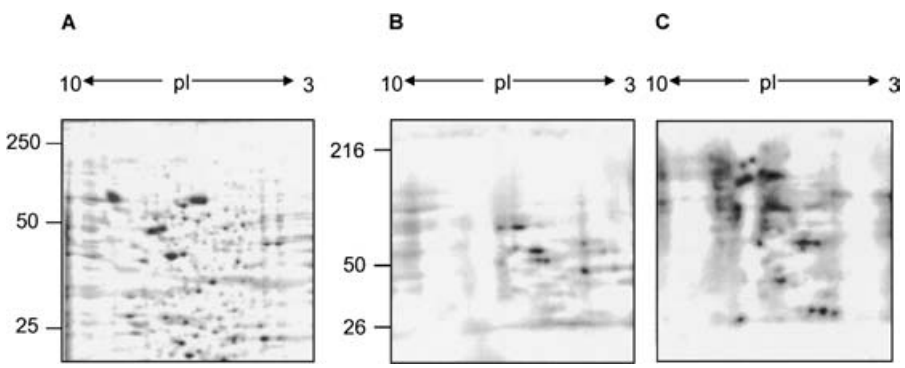

Figure 8 Reactivity of a subset of cellular proteins with biotinylated 15d-PGJ_2

(**A**) Cell lysate proteins from HEK-293 cells were separated by two-dimensional IEF and silver-stained. (**B**, **C**) Cell lysate proteins from HEK-293 cells, either untreated (**B**) or treated with 20 μM 15d-PGJ_2 (**C**), were separated by two-dimensional IEF, transferred on to nitrocellulose, and probed with streptavidin–HRP.

lysine residues. At this stage, we cannot exclude the possibility that the modification of other signalling molecules by electrophilic lipids contributes to the regulation of transcription. Rather, we postulate that this subproteome of proteins reactive to electrophiles can contribute a co-ordinated response to stress in the cell. In conclusion, we have shown for the first time that thiols in the Keap1 protein are targets of electrophilic lipid oxidation products, leading to the induction of ARE-dependent genes. We propose that this is one of the central mechanisms through which the ARE-dependent cytoprotective pathways are induced in concert during inflammation leading to adaptation and cytoprotection.

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