Available online at www.sciencedirect.com
SCIENCE DIRECT®
Biochemical and Biophysical Research Communications 320 (2004) 123–130
BBRC
www.elsevier.com/locate/ybbrc

# Identification of carbonylated proteins by MALDI-TOF mass spectroscopy reveals susceptibility of ER ☆

K. England* and T. Cotter

*Department of Biochemistry, Biosciences Institute, University College Cork, Cork, Ireland*

Received 19 May 2004
Available online 11 June 2004

**Abstract**

Reactive oxygen species are produced by metabolism over time, but can also be produced in more acute conditions of cell stress such as treatment with cytotoxic drugs. Treatment of HL-60 cells with peroxide results in cell death and protein carbonylation, a non-enzymatic protein modification that typically results from oxidative stress within cells. It has recently become clear that protein carbonylation during ageing is confined to specific proteins. It is therefore of interest to be able to identify which proteins are susceptible to protein carbonylation. Here we demonstrate immunoprecipitation of carbonylated proteins coupled with 2D-gel electrophoresis to identify carbonylated proteins by MALDI-TOF m/s fingerprinting. The results show that some ER proteins are readily carbonylated in response to peroxide treatment of HL-60 cells. This is likely to have implications for the induction of cell death in such cells.


*Keywords:* Carbonylation; Endoplasmic reticulum; Chaperone proteins; Reactive oxygen species

Reactive oxygen species (ROS) are constantly being generated within cells by metabolic processes [1–3]. ROS can also occur as the result of more acute cell stresses and may result in cell death via apoptosis or necrosis [4,5]. ROS include free radicals such as the superoxide anion ($O_2^{-}\cdot$), hydroxyl radicals' ($\cdot OH$), and hydrogen peroxide ($H_2O_2$), which are produced within the cell by the reduction of molecular oxygen by NADPH oxidase and superoxide dismutase. ROS can react with, and so damage, proteins, as well as lipids and carbohydrates. The resulting protein oxidation has been shown to be increased as cells age and reach senescence and so the study of protein oxidation has become an essential area in ageing research [6,7]. ROS have also been shown to be generated during treatment of cells with cytotoxic drugs such as VP16 [8] used in the treatment of cancer. It is as yet unclear how important the production of ROS by cytotoxic drugs is in the induction of cell death, but it is clear that ROS alone can induce cell death, possibly through the protein damage by post-translational modifications such as carbonylation.

Carbonylation is a non-reversible non-enzymatic modification of proteins that is typically the result of oxidative stress. ROS that can cause carbonylation of proteins include $H_2O_2$. Although just one of a number of possible oxidative modifications (oxidative stress can also cause direct modifications of protein side chains such as *o*-tyrosine, chloro-, nitrotyrosine, and dityrosine) carbonylation can be used as a marker for the identification of oxidised proteins [9]. Carbonylation is primarily associated with oxidative stress, however, it can also be caused by glycation or alkylating stress. Carbonyl groups can be formed by direct oxidation of amino acids (including histidine, lysine, arginine, threonine, and proline) [10] through Fenton Chemistry [11]. Amino acids can also be carbonylated through their reaction with the products of lipid peroxidation, such as 4-hydoxyl-2-nonenal [12–14], or through interaction with reducing sugars or their oxidation products. Until recently it was believed that protein carbonylation was

☆ *Abbreviations:* ACN, acetonitrile, DNP, dinitrophenol, DNPH, 2,4-dinitrophenyl hydrazine; ER, endoplasmic reticulum; ROS, reactive oxygen species; TBS, Tris-buffered saline, TFA, trifluoroacetic acid.

* Corresponding author.
*E-mail address:* k.england@ucc.ie (K. England).

0006-291X/$ - see front matter © 2004 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2004.05.144

random and ubiquitous, based on the understanding that the free radical attacks on proteins were uncatalysed. However, recent evidence has shown that this may not be the case and that in ageing, protein carbonylation occurs on only a few specific proteins. In the case of drosophilae, only aconitase and adenine nucleotide translocase showed any increase in carbonylation with age [15]. Cytochrome *c*, which is localised on the outer surface of the inner mitochondria where it is exposed to $H_2O_2$ and so may be expected to show evidence of oxidation, was shown not to be carbonylated [16]. Therefore, either cytochrome *c* is resistant to oxidation or any oxidised protein is rapidly degraded. In mouse plasma, ageing associated carbonylation was only seen in two proteins, albumin and transferrin [17], although different proteins were found in different species. In situations where protein carbonylation is due to more acute oxidative stresses there is evidence that actin is the most sensitive part of the cytoskeleton again supporting the idea that protein carbonylation is not a non-specific event [18,19]. It is therefore important that methods be established to identify which proteins are carbonylated. This is especially true if we are to understand the role of ROS mediated protein modifications such as protein carbonylation both by chronic cell stresses such as ageing and in situations of more acute cell stress such as during cytotoxic drug mediated cell death.

Although protein carbonylation has been known about and understood for some time research into identification of which proteins are carbonylated has been limited by technical difficulties [20]. These technical difficulties can be overcome through the use of proteomics to identify which proteins have been carbonylated [20,21]. Two dimensional gel electrophoresis followed by Western blotting is now being employed to look at protein carbonylation during ageing [20]. We show evidence here for a modification of this method such that the carbonylated proteins are immunoprecipitated and resolved by 2D gel electrophoresis. The resulting gel can then be stained with mass spectrometry compatible staining methods and carbonylated proteins excised and identified through techniques such as MALDI-TOF mass spectrometry.

## Materials and methods

*Chemicals and antibodies.* All chemicals were from Sigma (Dublin Ireland) unless otherwise indicated. The anti-caspase 3 antibody (Cell Signalling, Beverley, MA, USA) was used at 1/1000. The anti-actin antibody (Sigma) used for equal loading controls was used at 1/5000. The anti-DNP antibody (Dako, UK) was used at 1/5000. The phospho-eIF 2$\alpha$ antibody (Cell Signalling Technology, Beverley, MA, USA) was used at 1/1000. The anti-triose phosphate isomerase and phosphoglycerate mutase antibodies (Accurate Chemical and Scientific, NY, USA) were used at 1/1000. HRP-conjugated secondary antibodies were from Sigma.

*Tissue culture and induction of ROS.* HL-60 cells were maintained in RPMI 1640 supplemented with 10% foetal calf serum, 1% penicillin–streptomycin, and 2 mM L-glutamine (Gibco-BRL, Paisley, UK). Cells were maintained at 37 °C in a humidified 5% $CO_2$ atmosphere. For induction of apoptosis $5.0 \times 10^5$ cells/ml were incubated with peroxide at times and concentrations indicated.

*Detection of apoptosis.* Cell death was measured by incorporation of 50 μg/ml propidium iodide on a FACScan (Becton and Dickenson, UK) and on CellQuest software.

Cell death was qualified by morphological analysis of stained cytospin preparations. Rapi-diff (Diachem International, Lancashire, UK) stained centrifuged cell preparations were examined for the morphological characteristics of apoptosis. Apoptosis was quantified by counting three independent microscopic fields with at least 100 cells per field.

*DNPH modification of carbonylated proteins.* Cells ($5 \times 10^6$) were washed in 0.25 M sucrose and harvested in 100 μl of 40 mM Tris. This mix was sonicated and protein quantified using the Bio-Rad protein assay (Bio-Rad, UK) to obtain 100 μg of protein in 100 μl. Twenty microlitres of DNPH was added and the sample was incubated at room temperature for 30 min. Protein was then precipitated by the addition of an equal volume of ice cold 10% TCA on ice for 30 min. The protein pellet was then spun at top speed in a bench top centrifuge at 4 °C for 15 min. The sample was washed twice with acetone before resuspending in either IEF buffer (1 M thiourea, 8 M urea, 4% Chaps, and 2 mM tributylphosphine) for isoelectric focussing or 500 μl immunoprecipitation buffer (IP) (50 mM Tris–HCl, 0.5 mM EDTA, 2 mM EGTA, pH 7.5, 0.5% NP40, 10 μg/μl aprotinin, 10 μg/ml leupeptin, 2 mM AEBSF, 50 mM NaF, 5 mM Na pyrophosphate, and 10 μM Na orthovanadate). A probe sonicator was used to ensure complete resuspension.

*Immunoprecipitation.* Samples were pre-cleared by addition of 40 μl of protein A–Sepharose (PAS) slurry on an up and end stirrer at 4 °C for 2 h. After centrifugation to remove the PAS, the supernatant was transferred to a new tube and 10 μl of anti-DNP antibody was added. After mixing on an up-and-end stirrer overnight at 4 °C, 40 μl PAS slurry was added and the sample was mixed for a further 2 h. The sample was centrifuged at 8000*g* for 5 min in a bench-top centrifuge was washed twice in IP buffer before resuspending in IEF buffer.

*2D gel electrophoresis.* For whole cell analysis 50 μg protein was used, or for IPs the pellet was resuspended in 1 M thiourea, 8 M urea, 4% Chaps and 2 mM tributylphosphine with the addition of the appropriate carrier ampholytes (pH 3–10; Amersham Biosciences, UK). PAS was removed by centrifugation. Isoelectric focusing was carried out on an IPG Phor using 7 cm, pH 3–10, Immoboline Dry Strip Gels (Amersham Biosciences) using a gradient from 500 to 5000 V to give a total of 8750 Vh. After isoelectric focusing gel strips were incubated for 14 min in 50 mM Tris, pH 8.8, 6 M urea, 30% (v/v) glycerol, 2% SDS, 2% DTT, then for 4 min in 50 mM Tris, pH 8.8, 6 M urea, 30% (v/v) glycerol, 2% SDS, and 2.5% iodoacetamide prior to resolving by SDS–PAGE on 10% acrylamide gel.

*Western blotting.* Protein was transferred to nitrocellulose using standard procedures. The blot was blocked in 2% BSA in TBS/0.2% Tween. The blot was incubated in anti-DNP antibody in 2% BSA/TBST overnight, or other antibodies as appropriate, washed 4 times in TBST, and incubated for 30 min in anti-rabbit HRP antibody. Protein was detected using ECL (Amersham Biosciences).

*Gel staining and analysis.* For Coomassie staining gels were stained with colloidal blue Coomassie (Sigma, UK) according to manufacturer's instructions. Gels and 2D Western blots were scanned on a Epson Expression 1600 pro scanner and analysed using Phoretix 2D-gel analysis programme (NonLinear Dynamics). At least four duplicate gels were matched and analysed for each time point.

*Peptide mass fingerprinting.* For mass spectrometric analysis Spots were homogenised and destained by washing in 25 mM ammonium

bicarbonate/50% acetonitrile (ACN), dried in a vacuum centrifuge, and resuspended in 0.05 μg/μl Trypsin (Roche, UK) in 25 mM ammonium bicarbonate/5 mM CaCl. Spots were digested at 37 °C overnight. Peptides were eluted in 50% ACN/5% TFA and concentrated in a vacuum centrifuge. Salt was removed using ZipTips (Millipore, UK).

*MALDI-TOF mass spectrometry.* Mass spectrometry was carried out using a Voyager-DePro Biospectrometry workstation and Voyager Software (Applied Biosystems, UK). MALDI-TOF mass/spectrometry was performed in reflector mode and a multipoint calibration was carried out. Spectra obtained were matched through ExPasy PeptIdent (http://www.expasy.ch) and Protein Prospector MS-Fit (http://www.prospector.ucsf.edu).

## Results

### *Peroxide induced cell death in HL-60 cells*

HL-60 cells were treated with 8 μM to 80 μM peroxide for 12 h, and cell death was measured using propidium iodide analysis (Fig. 1A). Eight hundred micromolar peroxide killed approximately 50% of cells in 7 h and this concentration was selected for further studies. To confirm that cell death induced by peroxide was due to

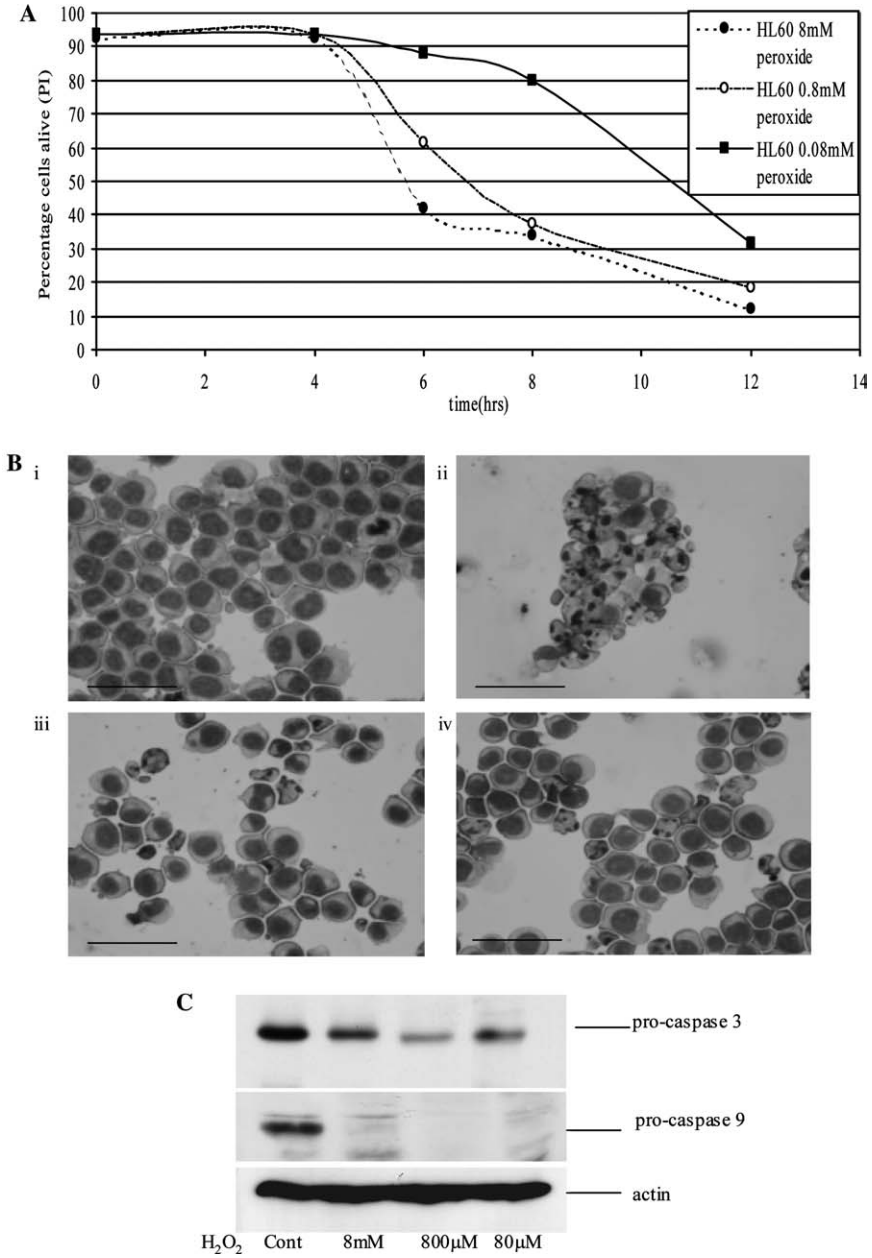


Fig. 1. $H_2O_2$ treatment of HL-60 cells results in induction of apoptosis. (A) HL-60 cells were treated with varying concentrations of peroxide and cell death was measured using propidium iodide staining over time. (B) Cytospin analysis was carried out on cells after 4 h treatment with (i) untreated control, (ii) 8 mM peroxide, (iii) 800 μM peroxide, and (iv) 80 μM peroxide, to demonstrate morphology was typical of an apoptotic and not necrotic induction of cell death. Scale bar represents 50 μm. (C) Western blots demonstrating cleavage of pro-caspase-3 and 9 after 3 h with various concentrations of peroxide.

apoptosis and necrosis, samples were analysed for the morphological characteristics of apoptosis such as nuclear fragmentation, membrane blebbing, and cell shrinkage (Fig. 1B) and for caspase cleavage (Fig. 1C). Both methods demonstrate that all concentrations of peroxide used kill cells through apoptosis as indicated by both morphology and cleavage of pro-caspase 3 and 9 (as shown by a reductions in the pro-caspase form of the enzyme). Peroxide induced cell death can be inhibited by pre-treatment of cells with 0.5 mM of the anti-oxidant *N*-acetylcysteine (data not shown).

*Detection of protein carbonylation*

Carbonylation of a protein may be readily detected by the reaction of carbonyl groups with 2,4-dinitrophenyl hydrazine (DNPH) to form hydrazones which are detected by the use of antibodies against DNP (2,4-dinitro hydrazones) or quantitated spectrophotometrically [20,22]. Induction of protein carbonylation in HL-60 cells following 800 μM peroxide treatment is shown in Fig. 2A. Carbonylation is induced rapidly and can be shown to be above control levels 2 h after treatment. The use of antibodies generated to DNP to detect protein carbonyls has already been demonstrated [20]. Fig. 2B shows Western blot analysis of protein carbonylation in response to peroxide treatment in HL-60 cells. However, this approach does not allow sufficient resolution of carbonylated proteins to allow for their identification. We therefore resolved proteins by 2D gel electrophoresis prior to immunoblotting with an anti-DNP antibody, Fig. 2C. This result demonstrates carbonylation of individual proteins in response to peroxide treatment. Nakamua and Goto [20] used 2D gel electrophoresis to detect protein carbonylation in tissue samples and found over 25 proteins showed positive DNP reactions. Although 2D gel electrophoresis gives good resolution of carbonylated proteins problems remain with identifying the carbonylated proteins as spots that reacted with the anti-DNPH antibody did not always correspond to Coomassie stained spots suggesting that protein carbonylation may in some way alter the resolution of protein by 2D gel electrophoresis [20]. We encountered similar problems using this approach. However, using gel matching we were able to identify some major carbonylated proteins, including actin, which has previously been reported to be a target of protein carbonylation. In order to identify some of the remaining carbonylated proteins we developed an immunoprecipitation method for DNPH modified proteins. This method is shown schematically in Fig. 3A. Controls for this reaction are shown in Fig. 3B, including a non-specific antibody control and a mock DNP treatment control. Once DNPH modified proteins from 800 μM $H_2O_2$ treated HL-60 cells were immunoprecipitated they were resolved by 2D-gel electrophoresis. These gels were then Coomassie stained for MALDI-TOF m/s

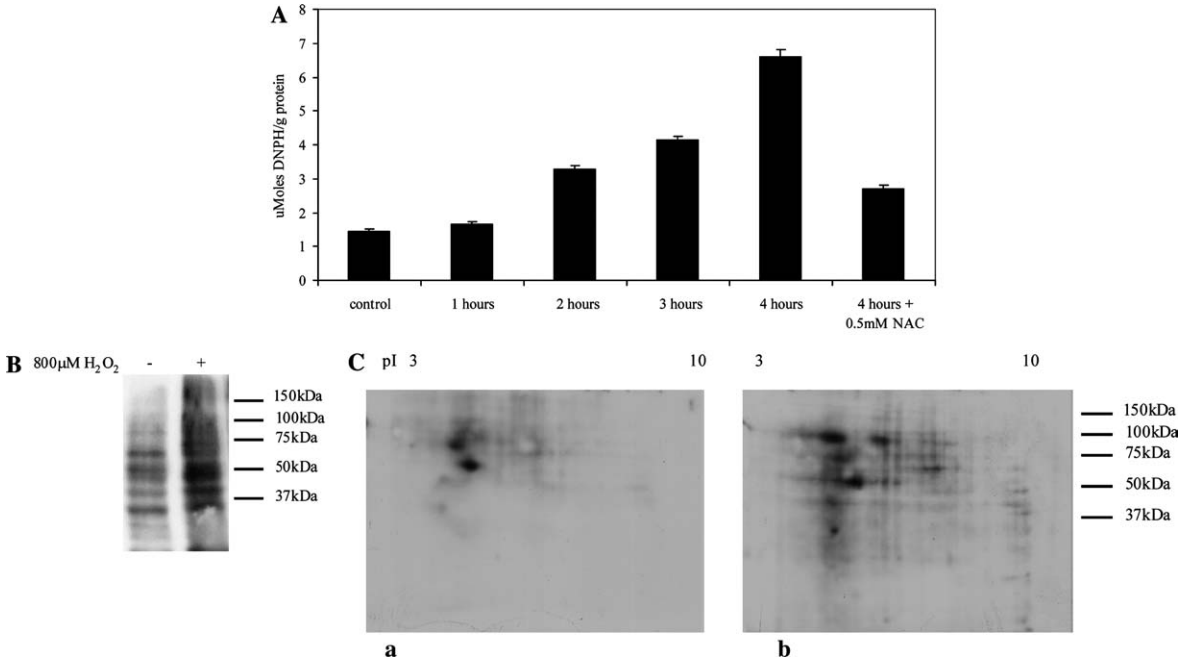


Fig. 2. $H_2O_2$ treatment of HL-60 cells results in increased protein carbonylation. (A) Induction of protein carbonylation over time after treatment of cells with 800 μM peroxide. Protein carbonylation was measured spectrophotometrically as micromolar DNPH per gram of protein. Protein carbonylation can be reduced by pre-treatment with 0.5 mM NAC. (B) Western blot showing induction of protein carbonylation in response to 800 μM peroxide treatment of HL-60 cells after 4 h. Total cell protein was extracted, modified with DNPH, and resolved by SDS–PAGE. After transfer to nitrocellulose the blot was probed with an anti-DNP antibody. (C) HL-60 cells were treated ±800 μM peroxide for 4 h, protein was DNPH treated, and protein homogenates were resolved by 2D-gel electrophoresis. After transferring to nitrocellulose, blots were probed with an anti-DNP antibody.

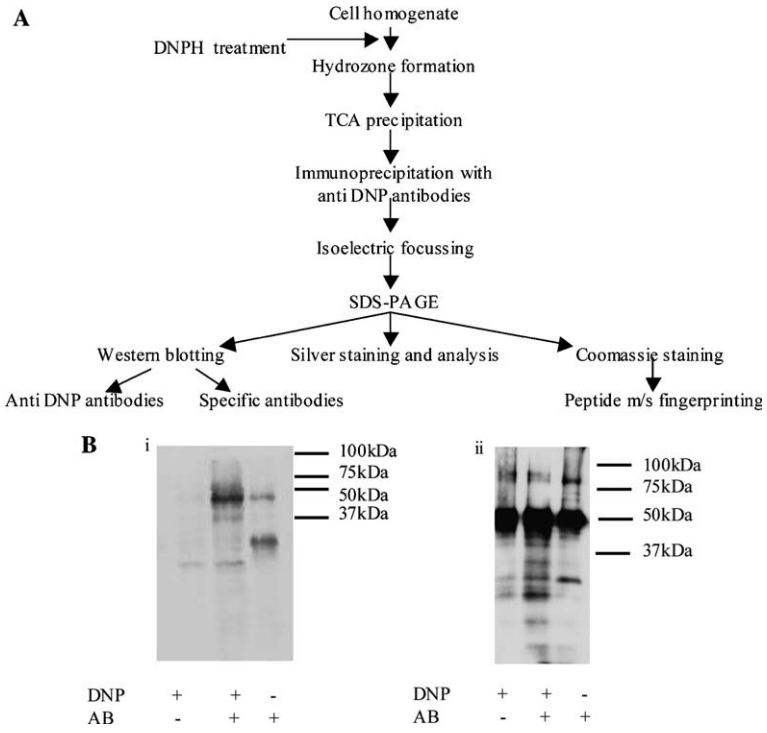


Fig. 3. Immunoprecipitation of DNP modified carbonylated proteins. (A) Schematic diagram showing method to immunoprecipitate DNPH modified carbonylated proteins for identification by MALDI-TOF m/s peptide mass fingerprinting, or other methods. (B) Controls for immunoprecipitation of DNPH modified carbonylated proteins; (i) show a Coomassie stained gel of immunoprecipitates of proteins from mock DNP treated, DNP treated and pulled down with anti-DNP antibody, and DNP treated and pulled down with irrelevant antibody, (ii) show Western blot with an anti-DNP antibody of the same samples.

(Fig. 4A) to allow identification of immunoprecipitated proteins. Other gels were transferred to nitrocellulose and the blot was probed for protein carbonyls using an anti-DNP antibody. This allowed us to identify any spots that were immunoprecipitated in this reaction but not carbonylated (Fig. 4B). This would include any proteins that associated with carbonylated proteins.

*Endoplasmic reticulum and glycolytic proteins are readily carbonylated in peroxide treated HL-60 cells*

Table 1 shows a list of proteins identified to have undergone protein carbonylation in HL-60 cells following treatment with 800 μM peroxide for 4 h. These include a number of glycolytic and ER proteins. To confirm the identities of these proteins where possible specific antibodies were used to probe DNP immunoprecipitates, as shown for actin, triose phosphate isomerase, and phosphoglycerate mutase (Fig. 4C).

*Peroxide treatment of HL-60 cells results in induction of the unfolded protein response pathway*

Protein carbonylation typically results in misfolding of the proteins. The detection of misfolded proteins within the ER results in the activation of the unfolded protein response pathway, including phosphorylation of the translation factor eIF 2α. As shown in Fig. 5 all concentrations of peroxide treatment resulted in phosphorylation of eIF 2α after 3 h, demonstrating that this response has been activated. Normally this response results in selective translation of chaperone proteins for cell survival, however, we have demonstrated that several of these chaperone proteins are themselves carbonylated. This is likely to result in the induction of apoptosis.

## Discussion

Advances in the study of protein carbonylation have been limited through technology. With recent advances in 2D gel electrophoresis and mass spectrometry it is now possible to use proteomics to analyse 2D gels to identify proteins that are carbonylated. Our immunoprecipitation method coupled with 2D gel electrophoresis has several advantages over conventional methods used to study protein carbonylation. First, the immunoprecipitation of DNPH modified proteins means that we are working predominantly with carbonylated proteins. This means that it is easier to identify proteins as it has been previously shown that protein bound DNPH leads to changes in the charge of a protein, although it has minimal effects on the molecular weight as seen by

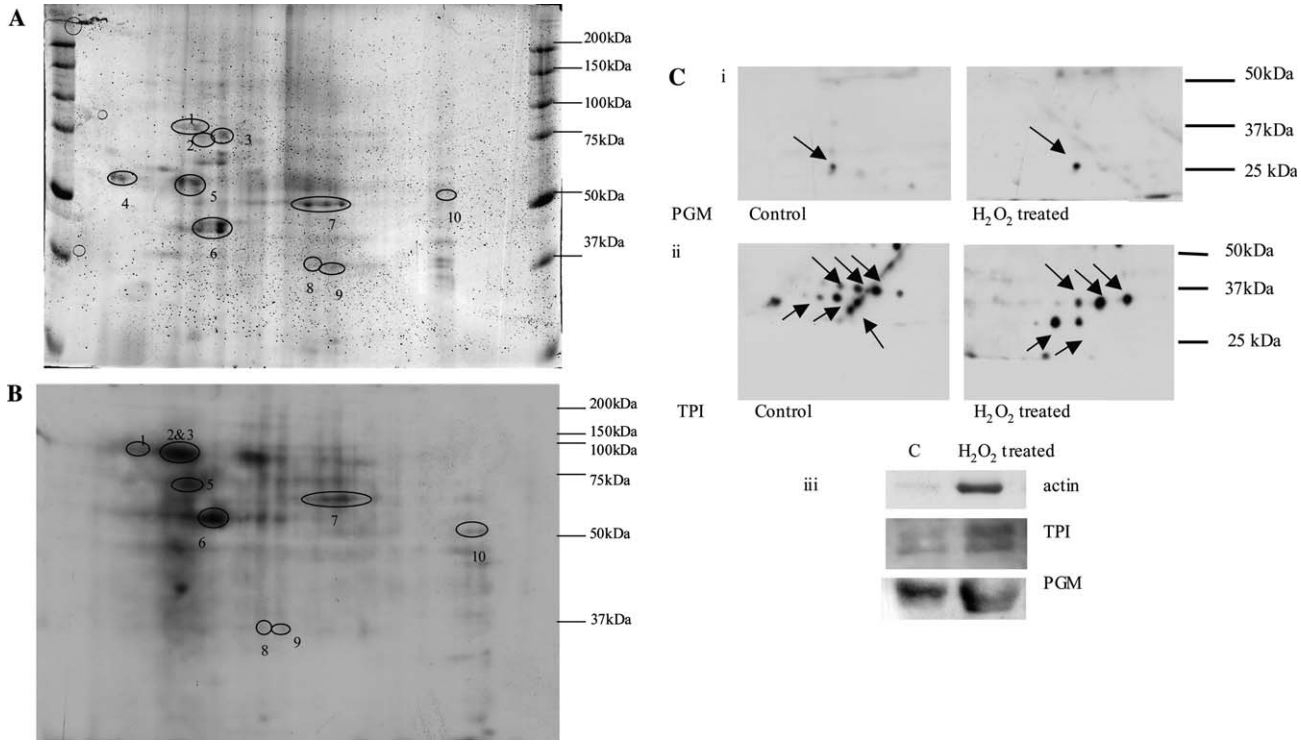


Fig. 4. Identification of carbonylated proteins in peroxide treated HL-60 cells. (A) Proteins from DNP immunoprecipitates from cells treated with 800 μM peroxide for 4 h were resolved by 2D-gel electrophoresis and Coomassie stained. Protein spots of interest, as indicated, were excised and processed for MALDI-TOF m/s fingerprinting. (B) Proteins from DNP immunoprecipitate from cells treated with 800 μM peroxide for 4 h were resolved by 2D-gel electrophoresis and immunoblotted with an anti-DNP antibody. (C) 2D Western blots of DNP immunoprecipitates from control and peroxide treated samples were probed using anti-phosphoglycerate mutase antibody (i) and anti-triose phosphate isomerase antibody (ii). DNP immunoprecipitates from control and peroxide treated samples were resolved by SDS–PAGE and probed with anti-actin, triose phosphate isomerase, and phosphoglycerate mutase antibodies to confirm m/s identifications of these protein.

Table 1
Proteins shown to be carbonylated were identified either through matching to master gels or through immunoprecipitation of DNPH modified proteins followed by peptide mass fingerprinting

| Spot No. | Protein | Function | Identification by | | |
|---|---|---|---|---|---|
| | | | Matching | m/s | Antibodies |
| 1 | HSP 90 | ER chaperone | | √ | |
| 2 | HSP 70 | ER chaperone | | √ | |
| 3 | Grp 78 | ER chaperone | √ | √ | |
| 4 | Calreticululin | ER calcium binding protein/chaperone | | √ | |
| 5 | Protein disulphide isomerase | ER chaperone | √ | √ | |
| 6 | Actin | Cytoskeleton | √ | √ | √ |
| 7 | α-Enolase | Metabolism | √ | √ | |
| 8 | Phosphoglycerate mutase | Metabolism | | √ | √ |
| 9 | Triose phosphate isomerase | Metabolism | | √ | √ |
| 10 | Fructose 5,6 bis phosphatase | Metabolism | | √ | |

Proteins identified as carbonylated in response to $H_2O_2$ treatment of HL-60 cells are shown, alongside details of how they were identified and their functions.

SDS–PAGE [23,24]. This has led to the development of methods to treat proteins with DNPH after isoelectric focussing and prior to SDS–PAGE [25]. Although this leads to increased ability to match carbonylated proteins detected by Western blotting by comparison to master gels, our method allows direct identification of carbonylated proteins. However, one potential advantage of DNPH treatment post-isoelectric focussing over the method described here is the possibility that proteins are lost in the TCA precipitation step. 2D gel electrophoresis allows good separation of proteins. This is essential for identification of proteins by peptide mass fingerprinting. We have also demonstrated that MALDI-TOF m/s peptide mass fingerprinting can be used to identify carbonylated proteins. This approach will be a powerful tool to advance our knowledge of protein carbonyla-

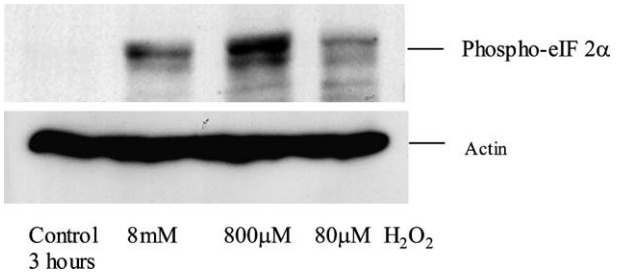


Fig. 5. Peroxide treatment induces eIF 2α phosphorylation. HL-60 cells were treated with peroxide for 3 h at the concentration shown. Western blots demonstrate an induction of eIF 2α phosphorylation with all peroxide treatments.

tion, especially given recent reports that carbonylation of proteins in ageing is not a random event, but instead is targeted to specific proteins. It is vital that we are able to determine which proteins are carbonylated in response to ageing or other stresses, to search for patterns to enable us to link protein carbonylation with changes in cell behaviour.

We have already observed that carbonylation of glycolytic proteins occurs in VP16 treated HL-60 cells, and that there is a corresponding decrease in the rate of glycolysis within those cells [8]. It is interesting to see that glycolytic proteins are also being carbonylated in response to peroxide treated cells. Metabolism is a major source of ROS production and inhibition of metabolic pathways may downregulate ROS. This would produce a feedback mechanism such that high levels of ROS result in a cell decreasing its ROS production. The cell may therefore benefit from the carbonylation of glycolytic proteins in response to ROS.

The observation that ER proteins are readily carbonylated is interesting given the importance of the ER in the induction of apoptosis. The ER is a site of ROS production and in contrast to the mitochondria has few anti-oxidant defences. It also has a highly redox environment and iron is present, both of which are important for protein carbonylation. As such the ER is likely to be especially susceptible to protein carbonylation and other oxidative stress mediated protein modifications. Indeed, Rabek et al. [26] recently demonstrated carbonylation of ER proteins in aged mouse liver. PDI, GRP78, and both HSPs are important chaperone proteins, important in detecting the presence of misfolded proteins in the ER. Carbonylation of these proteins is likely to inhibit the function of these proteins, and induce the unfolded protein response, as we have demonstrated through the induction of eIF 2α phosphorylation. Induction of the unfolded protein response can result in the induction of apoptosis [27]. ER stress can modulate apoptosis through a number of mechanisms. As yet these mechanisms are not well defined but the inactivation of these key ER proteins by carbonylation is likely to play a role in apoptosis.

## Acknowledgment

K. England is funded by Irish Cancer Research.

## References

[1] R.J Carmody, T.G. Cotter, Signalling apoptosis: a radical approach, Redox Rep. 6 (2) (2001) 77–90.
[2] J.T. Hancock, R. Desikan, S.J. Neill, Role of reactive oxygen species in cell signalling pathways, Biochem. Soc. Trans. 29 (1) (2001) 345–350.
[3] J.F. Curtin, M. Donovan, T.G. Cotter, Regulation and measurement of oxidative stress in apoptosis, J. Immunol. Methods 265 (1-2) (2002) 49–72.
[4] A. Gorman, A. McGowan, T.G. Cotter, Role of peroxide and superoxide anion during tumour cell apoptosis, FEBS Lett. 404 (1) (1997) 27–33.
[5] A.J. McGowan et al., Anti-oxidants and apoptosis, Biochem. Soc. Trans. 24 (1996) 229.
[6] R.L. Levine, Carbonyl modified proteins in cellular regulation, aging and disease, Free Radic. Biol. Med. 32 (9) (2002) 790–796.
[7] E.R. Stadtman, R.L. Levine, Protein oxidation, Ann. N Y Acad. Sci. 899 (2000) 191–208.
[8] K. England, C. O'Driscoll, T.G. Cotter, Carbonylation of glycolytic proteins is a key response to drug-induced oxidative stress and apoptosis, Cell Death Differ. 11 (3) (2004) 252–260.
[9] M. Chevion, E. Berenshtein, E.R. Stadtman, Human studies related to protein oxidation: protein carbonyl content as a marker of damage, Free Radic. Res. 33 (2000) S99–S108.
[10] B.S. Berlett, E.R. Stadtman, Protein oxidation in aging, disease, and oxidative stress, J. Biol. Chem. 272 (33) (1997) 20313–20316.
[11] G.T. Wondrak et al., Histone carbonylation in vivo and in vitro, Biochem. J. 351 (2000) 769–777.
[12] E.R. Stadtman , Role of oxidized amino-acids in protein breakdown and stability, in: Redox-Active Amino Acids in Biology, 1995, pp. 379–393.
[13] R.T. Dean et al., Biochemistry and pathology of radical-mediated protein oxidation, Biochem. J. 324 (1997) 1–18.
[14] E.R. Stadtman, B.S. Berlett, Reactive oxygen mediated protein oxidation in aging and disease, in: D.J. Gilbert (Ed.), Reactive Oxygen Species in Biological Systems, Kluwer Academic/Plenum Publishers, New York, 1999, pp. 657–675.
[15] N. Das et al., Selectivity of protein oxidative damage during aging in *Drosophila melanogaster*, Biochem. J. 360 (2001) 209–216.
[16] L.J. Yan, R.L. Levine, R.S. Sohal, Effects of aging and hyperoxia on oxidative damage to cytochrome *c* in the housefly. *Musca domestica*, Free Radic. Biol. Med. 29 (1) (2000) 90–97.
[17] C.K. Jana, N. Das, R.S. Sohal, Specificity of age-related carbonylation of plasma proteins in the mouse and rat, Arch. Biochem. Biophys. 397 (2) (2002) 433–439.
[18] I. Dalle-Donne et al., The actin cytoskeleton response to oxidants: from small heat shock protein phosphorylation to changes in the redox state of actin itself, Free Radic. Biol. Med. 31 (12) (2001) 1624–1632.
[19] I. Dalle-Donne et al., Actin carbonylation: from a simple marker of protein oxidation to relevant signs of severe functional impairment, Free Radic. Biol. Med. 31 (9) (2001) 1075–1083.
[20] A. Nakamura, S. Goto, Analysis of protein carbonyls with 2,4-dinitrophenyl hydrazine and its antibodies by immunoblot in two-dimensional gel electrophoresis, J. Biochem. 119 (1996) 768–774.
[21] M.A. Korolainen et al., Proteomic analysis of protein oxidation in Alzheimer's disease brain, Electrophoresis 23 (19) (2002) 3428–3433.
[22] R.L. Levine et al., Carbonyl assays for determination of oxidatively modified proteins, Methods Enzymol. 233 (1994) 346–357.

[23] J.M. Talent, Y. Kong, R.W. Gracy, A double stain for total and oxidized proteins from two-dimensional fingerprints, Anal. Biochem. 263 (1) (1998) 31–38.
[24] R.W. Gracy, J.M. Talent, A.I. Zvaigzne, Molecular wear and tear leads to terminal marking and the unstable isoforms of aging, J. Exp. Zool. 282 (1-2) (1998) 18–27.
[25] T. Reinheckel et al., Adaptation of protein carbonyl detection to the requirements of proteome analysis demonstrated for hypoxia/reoxygenation in isolated rat liver mitochondria, Arch. Biochem. Biophys. 376 (1) (2000) 59–65.
[26] J.P. Rabek, W.H. Boylston, J. Papaconstantinou, Carbonylation of ER chaperone proteins in aged mouse liver, Biochem. Biophys. Res. Commun. 305 (3) (2003) 566–572.
[27] R.V. Rao, H.M. Ellerby, D.E. Bredesen, Coupling endoplasmic reticulum stress to the cell death program, Cell Death Differ. 11 (4) (2004) 372–380.