UNIVERSITY OF BATH

Citation for published version:
Zhong, JL, Raval, CM, Nisar, MF, Bian, C, Zhang, J, Yang, L & Tyrrell, RM 2014, 'Development of refractoriness of ho-1 induction to a second treatment with uva radiation and the involvement of nrf2 in human skin fibroblasts', *Photochemistry and Photobiology*, vol. 90, no. 6, pp. 1340-1348. https://doi.org/10.1111/php.12343

DOI:
10.1111/php.12343

Publication date:
2014

Document Version
Peer reviewed version

Link to publication

This is the peer reviewed version of the following article: Zhong, JL, Raval, CM, Nisar, MF, Bian, C, Zhang, J, Yang, L & Tyrrell, RM 2014, 'Development of refractoriness of ho-1 induction to a second treatment with uva radiation and the involvement of nrf2 in human skin fibroblasts' Photochemistry and Photobiology, vol 90, no. 6, pp. 1340-1348., which has been published in final form at http://dx.doi.org/10.1111/php.12343. This article may be used for non-commercial purposes in accordance with Wiley Terms and Conditions for Self-Archiving

University of Bath

General rights
Copyright and moral rights for the publications made accessible in the public portal are retained by the authors and/or other copyright owners and it is a condition of accessing publications that users recognise and abide by the legal requirements associated with these rights.

Take down policy
If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

Download date: 21. Sep. 2019

Development of refractoriness of HO-1 induction to a second treatment with UVA radiation and the involvement of Nrf2 in human skin fibroblasts

Julia Li Zhong[1,2,3,*], Chintan M. Raval**, ChunXiang Bian[1], Jin Zhang[2], Li Yang[1], Rex M. Tyrrell[2]*

[1]The Base of "111 Project" for Biomechanics and Tissue Repair Engineering; Key Laboratory of Biorheological Science and Technology, Ministry of Education, Bioengineering College, Chongqing University, Chongqing 400044, China

[2]Department of Pharmacy and Pharmacology, University of Bath, Bath BA2 7AY, UK

[3]Dermatology Institute, Sichuan Provincial People's Hospital, Chengdu, Sichuan 610031, China

*Corresponding author e-mail:jzhonglyq@163.com (Julia Li Zhong)

prsrmt@bath.ac.uk (Rex M Tyrrell)

** Current address: Human Biosciences India Ltd.142P/143P, VasnaChacharwadi, Changodar, Ahmedabad - 382 213India

ABSTRACT

UVA treatment of cultured human skin fibroblasts (FEK4) has been shown previously to reduce transcriptional activation of hemeoxygenase 1 (HO-1) following a second dose of UVA radiation, a phenomenon known as refractoriness. This study demonstrates that the levels of HO-1 protein are also reduced after a second dose of UVA radiation as are Nrf2 levels, and there is less accumulation of Nrf2 in the nucleus whereas Bach1 does accumulate in the nucleus. Cell viability is further reduced and cell membrane damage increased as compared with a single UVA treatment when an initial UVA treatment was followed by a second dose. Knockdown of Nrf2 by siRNA (siNrf2) targeting caused additional refractoriness of HO-1 protein induction to a second UVA or heme treatment and this treatment also further enhanced cell damage by a second dose of UVA radiation. However, transfection with Nrf2 caused less refractoriness of HO-1 to a second dose of UVA and reduced cell damage by a second dose of UVA radiation. These findings are consistent with the proposal that Nrf2 is involved in HO-1 refractoriness and could serve as a cytoprotective factor against cell damage caused by repeated exposure to moderate doses of UVA radiation. We propose that protection by the Nrf2-HO-1 pathway protection may have clinical relevance since human skin is exposed repeatedly to UVA radiation.

35 **INTRODUCTION**

36 Ultraviolet A (UVA) radiation (320-400 nm) is a major part of solar ultraviolet light
37 (>90%) and causes an oxidative stress which has deleterious effects on human skin,
38 lead to erythema, photoaging and even cancer (1,2). Among antioxidant defence
39 mechanisms identified in skin is the enzymatic antioxidant, heme oxygenase 1 (HO-1,
40 Enzyme Classification Number: 1.14.99.3) (3), which catalyses heme to yield carbon
41 monoxide, biliverdin and iron (1,4). HO-1 is a sensitive marker of oxidative stress, and
42 is induced by UVA-irradiation of human skin fibroblasts (3) and many other cell types
43 (1,5). Induction of HO-1 gene expression involves transactivation by a bZIP
44 transcription factor Nrf2 (nuclear factor erythroid-2 p45-related factor 2) which forms
45 a heterodimer with Maf (small Maf family of proteins) and binds to the antioxidant
46 response elements (AREs) in the HO-1 gene promoter region. Conversely, Bach1 (BTB
47 and CNC homology-1), an additional bZIP factor, forms a Bach1-Maf complex and
48 functions as a repressor of HO-1 gene transcription (1,6,7). Our previous studies have
49 shown that UVA induces HO-1 gene expression, but does not alter HO-2 gene and
50 protein expression in human skin fibroblast (FEK4) cells (3,5). Treatment of chicken
51 embryo liver cells with heme leads to development of refractoriness to HO-1 gene
52 activation by a second heme treatment (8). Refractoriness of HO-1 gene induction to a
53 second dose of UVA radiation was also observed in FEK4 cells (9). However, the
54 refractoriness of HO-1 has not been examined at the protein level and the mechanism
55 of development of refractoriness is yet to be fully defined (8,9).

56 Nrf2 has been shown to play a pivotal role in preventing xenobiotic-related
57 toxicity and oxidative stress. The protective role of Nrf2 involves the induction of Phase
58 II detoxification enzymes as well as antioxidant enzymes, such as HO-1 through the
59 ARE (6,7). Nrf2 drive- HO-1 expression has been shown to be protective in human
60 lukemia as well as skin diseases (10,11). We have recently found that Nrf2 plays an
61 active role in both UVA radiation and heme induced HO-1 induction, and loss of Nrf2
62 sensitises FEK4 cells to UVA-radiation induced membrane damage as reflected by

63 enhanced LDH release (12,13). Bach1 plays a negative role in HO-1 induction in both
64 skin keratinocytes and fibroblasts (13-15). Since induction of HO-1 following UVA
65 irradiation is modulated by Nrf2 and Bach1 proteins, both therefore contribute to the
66 balance of cellular redox status (1,15).

67 In this study, we investigate if there is a refractoriness to HO-1 protein induction
68 corresponding to the refractoriness to HO-1 mRNA accumulation. In addition, we
69 investigated the role of Nrf2, an upstream transcriptional activator of the HO-1 gene, in
70 refractoriness and undertook a preliminary study of the role of Bach1 in the
71 refractoriness of HO-1. Our results are consistent with a link between Nrf2 and HO-1
72 refractoriness and demonstrate that deficiency of Nrf2 protein further increases cell
73 damage by a second dose of UVA irradiation. We also have some evidence that Bach1
74 is also likely to be involved in HO-1 refractoriness. These results provide a better
75 understanding of the pathophysiological effects of UVA irradiation on human skin cells,
76 and once similar studies are available *in vivo*, will help to evaluate the effects of
77 repeated physiological exposures of the skin to UVA radiation as well as repeated
78 clinical UVA phototherapy.
79

80 **MATERIALS AND METHODS**

81 **Cell Culture and Antibodies:**Human primary skin fibroblasts (FEK4) cells were
82 grown in Earle's modified Minimal Essential Medium (MEM). The MEM medium was
83 enriched with 15% (v/v) FCS and 2 mM*L*-glutamine along with 50 units/mL of
84 penicillin and streptomycin mixture. Antibodies against HO-1 (OSA-110) and HO-2
85 (OSA-200) were purchased from the Bioquote International (UK).Three antibodies
86 (Anti-Nrf2 H300, sc-13032; anti-Bach1 C-20, sc-14700; anti-actin sc-9104) and three
87 secondary antibodies (anti-goat, -rabbit and -mouse IgG) made by Santa Cruz
88 Biotechnology (USA) and Sigma-Aldrich Chemical Co. (UK), respectively, were used
89 (12-15).

90 **Irradiation of Cells with UVA:** Sellas (Germany) made broad spectrum (4-kW) lamp
91 was used to irradiate cells following standard procedures (12,13) while non-irradiated
92 cells were used as a background control (sham = 0 kJ/m^2). After incubation of UVA-
93 irradiated cells for a period of time (48-72 h), the cells were re-irradiated, and then
94 incubated in a saved conditional medium (cMedium) for the required time. The basic
95 protocol for the re-dosing regimen is set out in Table 1.

96

97 **Table 1.The basic protocol for the re-dosing regimen interval time (24-72h)**

1st dose(1°)	2^{nd}dose (2°)	Symbol
Sham	Sham	Sham(-/-)
Sham	UVA	-/UVA (-/+)
UVA	Sham	UVA/- (+/-)
UVA	UVA	UVA/UVA (+/+)

98 <Table 1>

99 **RNA Interference by siNrf2:** Sequences of all small interference RNAs (siRNAs)
100 against Nrf2 are as follow:

101 No.1 s9491 Sense: 5'-GAAUGGUCCUAAAACACCAtt-3'

102 Antisense: 5'-UGGUGUUUUAGGACCAUUCtg-3'

103 No.2 s9493 Sense: 5'-CAGUCUUCAUUGCUACUAAtt-3'

104 Antisense: 5'-UUAGUAGCAAUGAAGACUGgg-3'

105 Cells were transfected with two different concentrations of siNrf2 (5 and 30 nM) and
106 scrambled control (Sb, 30 nM) at the time of plating as follow: After trypsinization,
107 cells in suspension were transfected with scrambled oligonucleotides control (Sb,
108 AM4611) (Ambion) and siNrf2 using the siPORT™ NeoFX™ Transfection Agent
109 (AM4511, Ambion) using the different steps. The siRNA dilution were made in 100 µl
110 OPT-MEM and 5 µl NeoFX in 100 µl OPT medium respectively, uniformly mixed and
111 incubated together at room temperature (RT) for 10 min until the siRNA complexes are
112 formed. The siRNA complex was taken in 6- cm plates and a medium containing $3x10^5$
113 cells were added to make a final volume of 2.5 ml. After about 24 hours, the plates were
114 additionally given 1 ml of 15% (v/v) of FCS-MEM fresh medium and again incubated
115 for 48-72 h prior to further treatment (12-15).

116 **Transient transfection:** Cells were seeded into 96-well and 6- cm plates (in order to
117 perform RT-PCR of HO-1 and confirm transfection of Nrf2) 40 h before transfection
118 to reach 60% confluency. They were then transfected with pcDNA3.1-Nrf2 and its
119 control vector using the transfection reagent Lipofectamine 2000 (Invitrogen), in a 1:2
120 volume ratio (DNA:Lipofectamine) at RT according to the manufacturer's instructions.
121 The DNA–Lipofectamine complex was incubated with cells in Optimum (OPT)
122 medium (Invitrogen) for 6 h and then 1:1 growth medium was added for 6 h, finally
123 this medium was replaced with growth medium for a further 34 h (total 40 h) before
124 UVA irradiation (12, 15).

125 **RNA isolation, reverse transcription, and quantitative real-time PCR:** Total RNA
126 was collected from sham- and UVA-irradiated cultured skin cells using an RNA
127 extraction kit, following the supplier's instructions. RNA samples were quantified and
128 reverse transcription was performed (15). Quantitative real-time PCR primers were as
129 follows: HO-1, forward (F): AAGAGGCCAAGACTGCGTTC; reverse (R),
130 GGTGTCATGGGTCAGCAGC; Nrf2, F: GCGACGGAAAGAGTATGAGC R:

131 GTTGGCAGATCCACTGGTTT; GAPDH, F: GACATCAAGAAGGTGGTGAA; R,
132 TGTCATACCAGGAAATGAAG. RT-PCR was carried out with a Roche LightCycler
133 1.5 instrument using the SYBR green assay (Roche). A standard curve was created
134 using serial dilutions of a pooled sample of cDNA. Gene expression levels are presented
135 as arbitrary units normalized to the expression of the housekeeping gene GAPDH (15).
136 **Treatment of Cells with Heme:** Cells in the presence of hemin (ferri-protoporphyrin
137 IX) were incubated in cMedium for 1 h to generate heme (ferrous-protoporphyrin IX).
138 Cells were rinsed with PBS two times, and then cMedium was added and the cells
139 incubated for the next 48 h. Cells were re-treated again with hemin for 1 h, washed
140 twice using PBS and re-incubated with cMedium for the prescribed intervals.
141 To reduce the variations due to cell division, the freshly confluent FEK4 cells
142 (95-100%) were employed as described previously (9): Cells were treated with UVA
143 radiation, heme (1 h) or cadmium (1 h), re-incubated in cMedium for the indicated times
144 (interval times) and then were exposed to a second treatment of these agents.
145 **Protein extraction and western blotting:** Standard methods (12-14] were used to
146 extract proteins from the harvested cells following treatment. Equal amounts of total
147 protein lysate (30-50 μg depending on the experiment) and loading buffer were mixed
148 to separate on a 10% SDS-PAGE. A second gel was run using the identical protein
149 lysate as a loading control. After separated proteins were shifted onto PVDF (Millipore)
150 membranes and probed with actin (1:3000), Nrf2 (1:200), Bach1 (1:400) and HO-1
151 (1:200) antibodies, following a standard protocol (12-14). Subsequently,
152 chemiluminescence makes the protein bands visible on X-ray film by using the ECL
153 Western blotting detection system (Invitrogen). Digital densitometry was done to
154 quantify the intensity of protein bands by using the program NIH Image J1.33. Actin
155 was used to compare and normalize the data with respective controls and presented as
156 the fold change adjusted to 1.
157 **Immunocytochemistry:** Cells were grown to sub- or just freshly confluent on glass
158 cover-slips, collected and rinsed with PBS following fixation in 4% (w/v)

159 paraformaldehyde then incubated in 100% methanol at -20°C. The Image-iT™ Fx
160 signal enhancer (Invitrogen) was used to block the cells and then were treated with
161 1:100 Nrf2 and 1:200 Bach1 antibody and then Alexa-Fluor secondary antibody.
162 Hoechst nuclear stain was applied and the cover-slips mounted. For analysing the cells,
163 oil immersion epi-fluorescence Nikon Eclipse TE2000-U microscope was used and
164 finally the images were taken using UltraVIEW program.
165 **LDH measurement:** The levels of extracellular lactate dehydrogenase (LDH) were
166 monitored by using the ready to use cytotoxicity detection kit for LDH (Cat. No.
167 11644793001, Roche Applied Science) according to supplier instructions. Cells after
168 treatment with SiNrf2 (7500) were seeded into 96-well plates for 48 h, then irradiated
169 with UVA, incubate for 4 h and finally LDH release was measured according to Zhong
170 et al. (12). LDH release was calculated as the extracellular LDH as a fraction of total
171 LDH. Data was represented as the fold increase in LDH release over sham-irradiated,
172 scrambled siRNA or vector- treated control.
173 **MTS assay:** After treatment as described in the LDH assay, 20 µl of MTS reagent (The
174 Cell Titer 96® AQ_{ueous} Non-Radioactive Cell Proliferation Assay reagent Promega Cat.
175 No. G5421) were added to monitor the absorbance at 490 nm after the optimal time (1.5
176 h post incubation). The absorbance peaks, are the measure of the cell viability, were
177 used to compare sham-irradiated cells with that of scrambled siRNA or vector- treated
178 control, were set to 100.
179 **Statistical analysis:** Two-tailed T-test was applied to analyse the data and a *P*-value
180 <0.05 was taken to be statistically significant value. The descriptive statistics [mean,
181 standard errors (SE)] is presented graphically.
182

183 **RESULTS**

184 **HO-1 protein is induced by UVA irradiation**

185 HO-1 is an oxidative stress-inducible protein whose expression is highly inducible in
186 human primary skin fibroblast FEK4 and other cell lines, when treated with a large
187 number of physical stressors (e.g., UVA irradiation) and chemical (e.g., H_2O_2, hemin)
188 agents (1,3,16). HO-1 protein levels following the various doses of UVA irradiation in
189 FEK4cells has not examined before. To confirm the dose response for the inducibility
190 of HO-1 in FEK4 cells, we examined the protein levels of HO-1 by western blotting at
191 8 h following UVA irradiation in the range of 50 to 500 kJ/m^2. This study also included
192 longer incubation times (3 to 72 h) than a previous study (9). As expected, the induction
193 of HO-1 protein by UVA is dose-dependent. A maximal induction of HO-1 protein was
194 observed in FEK4 cells that were exposed to 250 kJ/m^2 UVA radiation. However, the
195 protein level declined with exposure to a higher dose of radiation (500 kJ/m^2) (Fig. 1A).
196 HO-1 protein induction post a moderate dose, i.e., 250 kJ/m^2 has not examined before,
197 so a time course of HO-1 protein induction was determined during 72 h following
198 treatment with a moderate dose (250 kJ/m^2) of UVA radiation. As shown in Fig. 1B,
199 the maximal increase of HO-1 protein (12±1.2 folds) was observed 12 h post irradiation
200 and HO-1 level remains higher than basal until at least 48 h and then decreased to basal
201 levels by 72 h.

202 <Figure 1>

203 **Refractoriness of HO-1 protein to re-induction occurs with UVA and heme but**
204 **not cadmium**

205 A Western blotting assay showed that HO-1 protein levels returned to the basal levels
206 60-72 h after single UVA treatment (Fig. 1). Total protein was collected between 8-12
207 h following the second treatment since during this period UVA radiation shows 8-12
208 fold HO-1 induction (Fig. 1B) (9,12,14). The levels of HO-1 protein were significantly
209 reduced in the pre-irradiated cells as compared with the non pre-irradiated samples
210 when the interval time was up to 48 h (Fig. 2A, middle panel, $P < 0.05$). Indeed, an
211 interval time of 72 h was required to obtain maximal reduction in HO-1 protein levels,

212 i.e. from 12 fold (-/UVA) to 6 fold (UVA/UVA) induction of HO-1 (Fig. 2A, right
213 panel, $P < 0.05$). No reduction of HO-1 induction was observed when the interval time
214 is 24 h (Fig. 2A, left panel, $P > 0.05$) and after an interval time of 96 h the HO-1
215 refractoriness is strongly reduced as compared with 72 h (data not shown). A second
216 treatment with heme induced a modest, yet significant reduction of HO-1 protein levels
217 as compared with cells that have not been pre-treated with heme, i.e., 9 fold vs. 6 fold
218 ($P < 0.05$, Fig. 2B). However, with a second cadmium (Cd) treatment or a cadmium pre-
219 treatment following by UVA radiation, HO-1 levels were not reduced (Fig. 2C). A
220 positive control demonstrated that the refractoriness response to UVA was normal i.e.,
221 10 fold (-/UVA) vs. 6 fold (UVA/UVA) at this 60 h interval time (Fig. 2B and C, P
222 < 0.05). After these treatments, HO-2 remains at a constant level (Fig. 2A and data not
223 shown).

224 <Figure 2>

225 **Refractoriness of Nrf2 activation to a second UVA treatment develops after an**
226 **initial UVA exposure**

227 Nrf2 has been implicated in the induction of HO-1 protein levels in skin fibroblasts by
228 both UVA radiation and heme (12). We hypothesise that the refractoriness to re-
229 induction of HO-1 protein by UVA might be associated with the altered Nrf2 and Bach1
230 accumulation in the nucleus following a second dose of UVA radiation. We thus
231 examined sub-cellular localisation and total protein levels of both Nrf2 and Bach1
232 following a second dose of UVA radiation. In order to perform immunostaining for the
233 localisation of Nrf2 and Bach1, a 48 h (but not 72 h) interval time was chosen between
234 two irradiations to avoid cells being too confluent and to facilitate cell morphology
235 studies. At this time (48 h), refractoriness to HO-1 mRNA accumulation was maximal
236 (9) but maximal refractoriness to HO-1 protein induction occurred a few hours later
237 (Fig. 2A). Both Nrf2 and Bach1 translocations were monitored at 2, 4 and 8 h following
238 a second dose of UVA irradiation and compared to levels observed after a single dose
239 of UVA radiation (Fig. 3). Fig. 3A showed that Nrf2 did not accumulate in the nucleus

240 at 2, 4 and 8 h following a second dose of UVA irradiation and it stays mostly in the
241 cytosol, whereas nuclear accumulation of Nrf2 did occur in cells that had not been
242 irradiated previously. Increased nuclear accumulation of Bach1 was observed following
243 a second spell of UVA irradiation (Fig. 3B).
244 Furthermore, Nrf2 protein levels in whole cell lysates were significantly reduced
245 following the second dose of UVA irradiation, i.e. 3 fold (UVA/UVA) vs. 5 fold (-
246 /UVA) Nrf2 induction 8 h following UVA (Fig. 3D); whereas Bach1 protein levels
247 were significantly higher, i.e. 3.3 fold (UVA/UVA) vs. 2 fold (-/UVA) (Fig. 3E). The
248 reduced Nrf2 and increased Bach1 levels corresponded temporarily with reduced HO-
249 1 levels (Fig. 3F). Reduced Nrf2 levels were also observed at the 72 h interval time
250 (data not shown) and these were comparable to those seen with the 48 h interval time.
251 The nuclear and cytosolic fraction were used and found that the Nrf2 levels are increase
252 even more in nuclear when compared with total cellular level (data not shown),but the
253 total cellular levels of Nrf2 reflect the nuclear increase of Nrf2, therefore was used for
254 the following experiments (12).
255 <Figure 3>
256 **Nrf2 may involve in the refractoriness of HO-1 protein to induction by a second**
257 **treatment with UVA irradiation and heme**
258 In a previous study, it has been shown that Nrf2 was involved in up-regulating HO-1
259 following UVA radiation and heme treatment and silencing of Nrf2 led to a reduction
260 in both UVA- and heme induced HO-1 levels (12,13). We studied whether modulation
261 of Nrf2 would effect a second treatment with either UVA radiation or heme with respect
262 to the refractoriness to induction of HO-1 protein in human dermal fibroblast FEK4
263 cells.
264 First silencing of Nrf2 was performed and Nrf2 protein knockdown by siRNA
265 was confirmed. Concentrations of 5 and 30 nM siNrf2 caused a reduction of up to 80%
266 of original protein levels (12). The level of HO-1 that is induced in cells, which had
267 been given siNrf2 were further reduced, i.e. from 7 fold of the scrambled control to 5

268 fold (5 nM siNrf2) and 2.5 fold (30 nMsiNrf2) 12 h following the second dose of UVA
269 radiation (Fig. 4A, *P* <0.05). Similarly, siNrf2 treatment has significantly decreased the
270 level of HO-1 induction by a second dose of heme (Fig. 4B, *P* <0.05). A second siNrf2
271 reagent that targeted a different exon in Nrf2 (Ambion, No. 2) showed similar results
272 (data not shown).

273 Nrf2 overexpression was confirmed and we observed that the concentrations of
274 0.5 and 2 μg Nrf2 caused an increase of up to 10-fold of basal mRNA levels (data not
275 shown). The level of induction of *HO-1* mRNA at 6 h following UVA irradiation (with
276 48 h interval time between irradiations) was shown to be 16- fold compared to the sham
277 control. The expression of HO-1 in cells, which had been transfected with Nrf2 was
278 increased, i.e. from 8- fold of the vector control to 11- fold (0.5 μg Nrf2) and 15- fold
279 (2 μg Nrf2) 6 h following the second dose of UVA radiation (Fig. 4C, P <0.05).

280 <Figure 4>

281 **Nrf2 may play a role in protection against a second treatment with UVA radiation**

282 Nrf2 is implicated in protection of many cell types against oxidative damage since it is
283 involved in up-regulating detoxifying phase-II enzymes, such as HO-1. A previous
284 study indicated that cells treated with UVA radiation twice, have the same extent of
285 free iron release as the first dose and thus may cause the same extent, if not more cell
286 membrane damage in FEK4 cells (17). We next examined whether loss of Nrf2 further
287 increased damage induced by a second UVA treatment, using LDH leakage and cell
288 viability to measure the damage. Our results show that a second UVA treatment
289 (UVA/UVA) significantly increased membrane damage to 2.6- fold (Fig. 5A, *P* <0.05)
290 and decreased cell viability to 79% (Fig. 5B, *P* < 0.05) when compared to the situation
291 in cells that had received a single dose of UVA (-/UVA). Furthermore, a second UVA
292 treatment caused both LDH leakage increase and cell viability loss and this damage was
293 further exacerbated by Nrf2 knockdown i.e., LDH leakage increased from 2.6- fold
294 (column 4) to 3- fold (column 6) and 3.6- fold (column 7) with 5 nM and 30 nM siNrf2
295 pre-treatment, respectively (Fig. 5A, *P* < 0.05). Also, cell viability decreased from 79%

296 (column 4) to 73% (column 6) and 65% (column 7) with 5 and 30 nM siNrf2 pre-
297 treatment, respectively (Fig. 5B, P <0.05). Both types of cell damage increase in a
298 siNrf2 concentration dependent manner.

299 We then tested whether an increase in Nrf2 levels reduced damage following a
300 second UVA treatment, using LDH leakage and cell viability to measure the damage.
301 Our results showed that the level of both increased LDH leakage (Fig. 5C) and cell
302 viability loss caused by a second UVA treatment (Fig. 5D) were reduced by Nrf2
303 overexpression i.e., LDH leakage reduction from 2.5- fold (vector control: column 3)
304 to 2.3- fold (column 4) and 1.9- fold (column 5) with 0.5 and 2 μg Nrf2 transfection,
305 respectively (Fig. 5C, P < 0.05), while a single UVA treatment caused a ~2- fold LDH
306 release when compared with sham control as observed previously (12). We observed
307 cell viability loss from 20% (column 3) to 15% (column 4) and 8% (column 5) with 0.5
308 and 2 μg Nrf2 transfection, respectively (Fig. 5D, P < 0.05), with a significant recover
309 in cell viability loss (P > 0.05). Both types of cell damage decrease in a Nrf2
310 concentration- dependent manner.

311

312 <Figure 5>

313

314

315 **DISCUSSION**

316 **Refractoriness of HO-1 protein following the second dose of UVA irradiation**

317 Refractoriness of HO-1 gene activation to a second treatment with hemin was first
318 observed in chicken embryo liver cells (8). Later it was shown that, following a second
319 treatment with a moderate, physiological dose of UVA irradiation or hemin, human
320 skin fibroblasts FEK4 had acquired refractoriness to activate the HO-1 gene as
321 monitored at the level of transcription (9). In this study we extended these observations
322 to the protein level by studying the development of refractoriness to re-induction of
323 HO-1 protein following a second treatment with UVA radiation at different time
324 intervals. As expected, refractoriness to HO-1 protein induction develops at later times
325 than refractoriness to HO-1 mRNA accumulation and the former occurs maximally with
326 a 48 h interval time. The initial UVA inducedHO-1 mRNA accumulation is back to
327 normal at 16 h following UVA irradiation (9). The maximal reduction in HO-1 protein
328 levels (refractoriness) occurred following the second dose of UVA irradiation when
329 cells were incubated for 72 h between two doses of UVA irradiation. The apparent lack
330 of refractoriness to HO-1 induction for UVA/UVA treatment at a 24 h interval may be
331 due to increased amount of HO-1 protein as observed in the relevant controls (UVA/-):
332 still remaining high level 24 h after irradiation, i.e. the pre-irradiated cells still had 3-4
333 times higher level of HO-1 protein as compared to the sham control. It is notable that
334 an interval time of 24 h was required to obtain a maximal reduction in re-induction of
335 HO-1 activity by a moderate dose of UVA irradiation in a mouse model (18) indicating
336 either a species, or *in vitro* versus *in vivo* difference.

337 Both UVA and heme treatment can cause refractoriness to induce HO-1 protein
338 by a second treatment. Cadmium chloride (cadmium) increased both HO-1 and Nrf2
339 levels (19) but it does not lead to the refractoriness to any type of second treatment.
340 This is similar to previous studies with sodium arsenite, another strong HO-1 inducer
341 and it is likely that this is because these compounds do not alter heme levels (1,9,15,20).
342 A combination of heme and UVA radiation treatments was not given because heme

343 sensitises cells against UVA mediated cell death (9,16). The UVA induction of HO-1
344 level is correlated to the extent of UVA released microsomal heme, and reduced heme
345 levels were found 48 h following UVA irradiation (16). Refractoriness observed
346 following a second treatment with UVA radiation or heme might link to the reduction
347 of heme levels because it is known that the first treatment strongly increases HO-1
348 levels(9). This result was supported by our previous study showing that the inhibition
349 of heme synthesis significantly reduced UVA-induced HO-1 protein levels (12,14).

350 **Refractoriness of HO-1 protein may due to Nrf2 refractoriness**

351 Nrf2 is involved in dissipating the stress and providing a protective response following
352 UVA induction of oxidized phospholipids in skin cells (21). Loss of Nrf2 increases
353 UVA-mediated apoptosis in mouse skin fibroblasts (22). The protein can be activated
354 as a result of stabilisation, translocation and nuclear accumulation (6). Our results show
355 that Nrf2 silencing leads to an enhanced refractoriness to HO-1 induction after a second
356 treatment with both UVA radiation and heme; and that an increase in Nrf2 protein leads
357 to a reduced refractoriness to HO-1 induction after a second treatment with UVA
358 radiation. These results indicate that pre-irradiation not only leads to reduced total
359 levels of Nrf2 protein accumulation following a second dose, but it may also result in
360 reduced Nrf2 nuclear accumulation. UVA treatment leads to both a reduced level of
361 Nrf2 activation, and increased Bach1 activation when a second dose of UVA is applied.
362 Reduction of Nrf2 leads to a lower induction of HO-1 by UVA irradiation (12) while
363 an increase of Nrf2 will lead to high induction of HO-1 by UVA irradiation. Conversely,
364 Bach1 reduction leads to increased levels of HO-1 after UVA irradiation in these cells
365 (15). The alteration of Nrf2 up-regulation by either heme or UVA irradiation may
366 therefore be involved in the HO-1 refractoriness response.

367 Both Nrf2 and Bach1 translocation and expression are modulated by UVA
368 radiation which therefore modulates UVA-induced HO-1 expression (12-15). We may
369 expect that lack of free heme following an increased activity of HO-1 will prevent
370 stabilisation of Nrf2. The consequent reduction in Nrf2, together with the stabilisation

371 and increase in Bach1 levels will both contribute to diminish HO-1 re-induction by a
372 second UVA dose.

373 **Repeated UVA irradiations cause more cell damage and Nrf2 is implicated in**
374 **protection from a second treatment with UVA irradiation**

375 Loss of Nrf2 sensitises cells to UVA radiation induced damage (12,22). Nrf2 is
376 involved in protecting many different cell lines against oxidative damage since it up-
377 regulates detoxifying phase II enzymes, especially HO-1. The protective effect of Nrf2
378 against UVA radiation as well as several other damaging agents has been demonstrated
379 in skin keratinocytes and fibroblasts (12,13,22). A previous study indicated that a
380 second regime of UVA radiation causes the same extent of free iron release as the first
381 dose and thus may cause similar or increased cell membrane damage in FEK4 cells (17).
382 These findings are in agreement with studies by Merwald (23), who found that the
383 fractioned UVA exposure precedes a greater rate of cells mortality compare with single
384 regime. However, they are in contrast to a previous finding from Tyrrell's laboratory
385 that fully confluent FEK4 cells are protected by pre-irradiation with an optimum dose
386 of UVA radiation (250 kJ/m^2) from high doses of UVA radiation (750 kJ/m^2) (24). In
387 the previous study, protection was found 24 h following UVA treatment, when HO-1
388 protein levels are significantly higher (9,24) (Fig. 1). Furthermore, fully confluent cells
389 are generally more resistant to UVA irradiation than less confluent cells.

390 A related study in a mouse model reported that UVA radiation induced refractoriness
391 to HO-1 induction by a second UVA treatment with a 24 h time interval and this is
392 linked to reduced immunoprotection (18). This result further indicated that repeated
393 UVA irradiation may cause more damage to skin when compared with a single
394 exposure. The fact that a second exposure with an optimum dose (250 kJ/m^2) of UVA
395 can cause and enhance cell damage relative to a single dose of UVA may be attributable
396 to a lower expression of Nrf2 protein under these conditions. Repetitive UVA damage
397 may be reduced by an increase in Nrf2 levels, which further implies that activation of
398 Nrf2 contributes to the protection of human skin fibroblasts against oxidative damage.

399 However other molecules may also be involved (12,13,25,26). It has been argued that
400 Nrf2 signalling may offer a protective role in aging, including photoaging and it may
401 play a role during keratinocyte differentiation (27,28). Further, increase in Nrf2 levels
402 by moderate proteasome activation may affect the aging process and the cellular
403 response to oxidative stress in human fibroblasts (29). While the Nrf2/HO-1 system
404 may protect human skin cells against UVA-mediated damage, the activation may
405 increase tumour progression (30).

406 In summary, we have demonstrated that UVA radiation causes refractoriness of
407 human skin fibroblasts to re-induction of both HO-1 and Nrf2 by a second dose of UVA
408 irradiation and that both Nrf2 and Bach1 might cooperate in HO-1 refractoriness. While
409 our data support the concept that Nrf2 may have a protective function in skin fibroblasts
410 upon single and multiple UVA treatments, the involvement of Nrf2 in human skin
411 protection *in vivo* remains to be determined. Repeated introduction of human skin cells
412 to moderate and high doses of UVA irradiation results in enhanced cell damage and
413 Nrf2 may offer protection under such conditions.

414 **ACKNOWLEDGMENTS**: This work has been supported by: National Natural
415 Science Foundation of China (81271776); and a project grant (BB/D521530/1) from
416 the UK Biotechnology and Biological Sciences Research council (BBSRC). We offer
417 special thanks are for Dr. KSG Parsons (Marietta College) for proof reading of this
418 manuscript and Dr. Yiguo Zhang and Mr. MF Nisar (Chongqing University) for useful
419 discussion.

420

421 **REFERENCES**

422 1. Tyrrell, R. M. (2004) Solar ultraviolet A radiation: an oxidizing skin
423 carcinogen that activates heme oxygenase-1. *Antioxid. Redox Signal*. 6,
424 835-840.

425 2. Tyrrell, R. M. (2012) Modulation of gene expression by the oxidative stress
426 generated in human skin cells by UVA radiation and the restoration of redox
427 homeostasis. *Photochem. Photobiol. Sci*. 11, 135-147.

428 3. Keyse, S. M. and R. M. Tyrrell (1989) Heme oxygenase is the major 32-kDa
429 stress protein induced in human skin fibroblasts by UVA radiation, hydrogen
430 peroxide, and sodium arsenite. *Proc. Natl. Acad. Sci. USA* 86, 99-103.

431 4. Maines, M.D., G. M. Trakshel and R. K. Kutty (1986) Characterization of two
432 constitutive forms of rat liver microsomal hemeoxygenase: only one molecular
433 species of the enzyme is inducible. *J. Biol. Chem*. 261, 411-419.

434 5. Applegate, L.A., A. Noel, G. F. Vile, E. Frenk and R. M. Tyrrell (1995) Two
435 genes contribute to different extents to the hemeoxygenase enzyme activity
436 measured in cultured human skin fibroblasts and keratinocytes: implications
437 for protection against oxidant stress. *Photochem. Photobiol*. 61, 285-291.

438 6. Higgins, L. G., M. O. Kelleher, I. Eggleston, K. Itoh, M. Yamamoto and J. D.
439 Hayes (2009)Transcription factor Nrf2 mediates an adaptive response to
440 sulforaphane that protects fibroblasts in vitro against the cytotoxic effects of
441 electrophiles, peroxides and redox-cycling agents. *Toxicol. Appl. Pharmacol*.
442 237, 267-280.

443 7. Zenke-Kawasaki, Y., Y. Dohi, Y. Katoh, T. Ikura, M. Ikura et al. (2007) Heme
444 induces ubiquitination and degradation of the transcription factor Bach1. *Mol.*
445 *CellBiol*. 27, 6962-71.

446 8. Srivastava, K.K., E. E. Cable, S. E. Donohue and H. L. Bonkovsky (1993)
447 Molecular basis for heme-dependent induction of hemeoxygenase in primary

448 cultures of chick embryo hepatocytes. Demonstration of acquired
449 refractoriness to heme. *Eur. J. Biochem*. 213, 909-917.
450 9. Noel, A. and R. M. Tyrrell (1997) Development of refractoriness of induced
451 human heme oxygenase-1 gene expression to reinduction by UVA irradiation
452 and hemin. *Photochem. Photobiol*. 66, 456-463.
453 10. Sgarbossa, A., M. Dal Bosco, G. Pressi, S. Cuzzocrea, R. Dal Toso and M.
454 Menegazzi (2012) Phenylpropanoid glycosides from plant cell cultures induce
455 hemeoxygenase 1 gene expression in a human keratinocyte cell line by
456 affecting the balance of NRF2 and BACH1 transcription factors.*Chem. Biol.*
457 *Interact*. 199, 87-95.
458 11. Rushworth, S. A. and D. J. MacEwan (2013) The role of Nrf2 and
459 cytoprotection in regulating chemotherapy resistance of human leukemia cells.
460 *Cancers*3, 1605-1621.
461 12. Zhong, J. L., G. P. Edwards, C. M. Raval, H. Li and R. M. Tyrrell (2010)
462 The role of Nrf2 in ultraviolet A mediated hemeoxygenase 1 induction in
463 human skin fibroblasts. *Photochem. Photobiol. Sci*. 9, 18-24.
464 13. Tian, F.F., F. F. Zhang, X. D. Lai, L. J. Wang, L. Yang, X. Wang, S. Gurinder
465 and J. L. Zhong(2011)Nrf2-mediated protection against UVAradiation in
466 human skin keratinocytes. *BioscienceTrends* 5, 23-29.
467 14. Raval, C. M., J. L. Zhong, S. A. Mitchell and R. M.Tyrrell(2012) The role of
468 Bach1 in ultraviolet A mediated human hemeoxygenase 1 regulation in
469 human skin fibroblasts.*Free Rad. Biol. Med*. 52, 227-36.
470 15. Zhong, J. L., C. M. Raval, G. P. Edwards and R. M. Tyrrell (2010) A role for
471 Bach1 and HO-2 in suppression of basal and UVA-induced HO-1 expression
472 in human keratinocytes.*Free Rad. Biol. Med*. 48, 196-206.
473 16. Kvam, E., A. Noel, S. Basu-Modak and R. M. Tyrrell (1999)
474 Cyclooxygenase dependent release of heme from microsomal hemeproteins

475 correlates with induction of hemeoxygenase 1 transcription in human
476 fibroblasts. *Free Rad. Biol. Med*. 26, 511-517.
477 17. Zhong, J. L. (2001) UVA-mediated iron release in skin cells. PhD Thesis
478 2001. University of Bath.
479 18. Reeve, V. E. and D. Domanski (2002) Refractoriness of UVA-induced
480 protection fromphotoimmunosuppression correlates with hemeoxygenase
481 response to repeated UVA exposure. *Photochem. Photobiol*. 76, 401-405.
482 19. Chen, J. and Z. A. Shaikh (2009) Activation of Nrf2 by cadmium and its role
483 in protection against cadmium-induced apoptosis in rat kidney cells. *Toxicol.*
484 *Appl. Pharmacol*. 241, 81-89.
485 20. Cooper, K. L., K. J. Liu and L. G. Hudson (2009) Enhanced ROS production
486 and redoxsignaling with combined arsenite and UVA exposure: contribution
487 of NADPH oxidase. *Free Rad. Biol. Med*. 47, 381-388.
488 21. Gruber, F., H. Mayer, B. Lengauer, V. Mlitz, J. M. Sanders, A. Kadl et al.
489 (2010) NF-E2-related factor 2 regulates the stress response to UVA-1-
490 oxidized phospholipids in skin cells. *FASEB J*. 24, 39-48.
491 22. Hirota, A., Y. Kawachi, K. Itoh, Y. Nakamura, X. Xu, T. Banno et al. (2005)
492 Ultraviolet A irradiation induces NF-E2-related factor 2 activation in dermal
493 fibroblasts: protective role in UVA-induced apoptosis. *J. Invest. Dermatol*.
494 124, 825-832.
495 23. Merwald, H., G. Klosner, C. Kokesch, M. Der-Petrossian, H. Honigsmann
496 and F. Trautinger (2005) UVA-induced oxidative damage and cytotoxicity
497 depend on the mode of exposure. *J. Photochem. Photobiol*. 79, 197-207.
498 24. Vile, G. F., S. Basu-Modak, C. Waltner and R. M. Tyrrell (1994)
499 Hemeoxygenase 1 mediates an adaptive response to oxidative stress in
500 human skin fibroblasts. *Proc. Natl. Acad. Sci. USA* 91, 2607-2610.
501 25. Vile, G.F. and R. M. Tyrrell (1993) Oxidative stress resulting from
502 ultraviolet A irradiation of human skin fibroblasts leads to a

503 hemeoxygenase-dependent increase in ferritin. *J. Biol. Chem*. 268, 14678-
504 14681.
505 26. MacLeod, A. K., M. McMahon, S. M. Plummer, L. G. Higgins, T. M.
506 Penning, K. Igarashi et al. (2009) Characterization of the cancer
507 chemopreventive NRF2-dependent gene battery in human keratinocytes:
508 demonstration that the KEAP1-NRF2 pathway, and not the BACH1-NRF2
509 pathway, controls cytoprotection against electrophiles as well as redox-
510 cycling compounds. *Carcinogenesis* 30, 1571-1580.
511 27. Sykiotis, G. P. and D. Bohmann (2010) Stress-activated Cap'N'Collar
512 transcription factors in aging and human disease. *Sci. Signal*. 3, 112-116.
513 28. Piao, M. S., J. J. Park, J. Y. Choi, D. H. Lee, S. J. Yun, J. B. Lee and S. C.
514 Lee (2012) Nrf2-dependent and Nrf2-independent induction of phase 2
515 detoxifying and antioxidantenzymes during keratinocyte differentiation. *Arch.*
516 *Dermatol. Res*.304, 387-395.
517 29. Kraft, D. C., C. C. Deocaris, R. Wadhwa and S. I. Rattan (2006) Pre-
518 incubation with the proteasome inhibitor MG-132 enhances proteasome activity
519 via the Nrf2 transcription factor in ageing human skin fibroblasts. *Ann. N.Y.*
520 *Acad. Sci*. 1067, 420-424.
521 30. Na, H. K., Y. J. Surh (2014). Oncogenic potential of Nrf2 and its principal
522 target protein heme oxygenase-1. Free Radic Biol Med. 67:353-65.
523

524 **FIGURE CAPTIONS**

525 **Figure 1. Dose and time dependence of induction of HO-1 protein by UVA in the**
526 **human skin fibroblastFEK4.** Cell lysates were harvested at 8 h after UVA radiation
527 (A), or at the indicated times following exposure to 250 kJ/m2UVA radiation (B). In
528 each lane, total protein (40 μg) was separated on 12% SDS-PAGE gels, transferred to
529 PVDF membranes and probed for HO-1 and actin. Quantification of optical densities
530 of individual bands was carried out using digital densitometry. Values were normalised
531 with respect to the actin signals. The expression levels of HO-1 are shown as a fold
532 induction relative to the sham irradiated control (set to 1) on the bar graph. Data are
533 presented as mean ± S.E (n = 4). *, P<0.05， **, P<0.01 vs. the sham control.

534

535 **Figure 2. Treatments with UVA irradiation and heme, but not cadmium lead to**
536 **refractoriness to re-induction of HO-1 protein.** Cells were treated with 250 kJ/m^2
537 UVA irradiation (A), 5 μMheme (B) or 2.5 μM cadmium (C) for 1 h, and then incubated
538 in conditional medium (cMedium) for 24, 48, 72h (Figure 2A,UVA irradiation), and 60
539 h (Figure 2B and C, heme or cadmium) before a second treatment under similar
540 conditions. The cells were harvested at 12 h after second treatment and assayed for the
541 expression of HO-1 protein. Values were normalised by actin loading, the levels of HO-
542 1, a relative fold induction to the sham irradiated control (set to 1) were shown in the
543 bar graph. Data are presented as mean ± SE (n = 4). **P*<0.05**,** ***P*<0.01 vs. the sham
544 control. Cd: cadmium. 1°: first treatment, 2°: second treatment.

545

546 **Figure 3.The effect of a second UVA radiation treatment on total and nuclear**
547 **accumulation of Nrf2 and Bach1 protein.**FEK4 cells were pre-treated with 250 kJ/m^2
548 UVA irradiation, and re-incubated in cMedium for 48 h, followed by a second dose of
549 UVA. Cells were collected at the indicated times post irradiation. A. Cells grown on
550 coverslips were collected and fixed, then permeabilised and immunostained with anti-
551 Nrf2 antibody (1:200, green: 3A), Bach1 (1:300, Red: 3B). Hoechst dye (shown in red)

552 was used to visualise cell nuclei. 3C.Western blotting was performed with 35μg/lane of
553 whole cell protein as described previously; Actin signals were determined for all
554 samples and used to normaliseNrf2 Bach1 and HO-1, protein levels. Relative Nrf2 (3D),
555 Bach1 (3E) and HO-1 (3F), levels in the samples were expressed as fold increases. Data
556 are presented as mean ± SE (n = 4). * P<0.05vs.the relevant single irradiated control (-
557 /UVA).
558

559 **Figure 4. Effect of modulation of Nrf2 levels on refractoriness of HO-1 to induction**
560 **by a second treatment with UVA.** FEK4 cells were transiently transfected with
561 vehicle control (–), negative scrambled control siRNA (Sb, 30 nM), 5 and 30 nM of
562 siNrf2, using siPORTNeoFX transfection reagent (Ambion) as described in materials
563 and methods (A and B). Cells were transiently transfected with vector control (V), Nrf2
564 constructs (0.5 and 2 μg), using lipofectamine transfection reagent as described in
565 materials and methods (C).
566 Cells were cultured for 60 h, then pre-treated with 250 kJ/m^2 UVA irradiation (A) or 5
567 μM heme (B), and re-incubated in cMedium for an additional 60 h, followed by a
568 second dose of either 250 kJ/m^2 UVA irradiation or heme, and collected 12 h later;
569 Cells were transfected with Nrf2 as described in Fig. 4, then pre-treated with 250 kJ/m^2
570 UVA irradiation, and re-incubated in cMedium for an additional 48 h, and collected
571 at 6 h following UVA irradiation (C). Western blotting of HO-1 protein and PCR for
572 mRNA was performed as described previously. Relative HO-1 levels were expressed
573 as a fold increase after being normalised by actin protein (A and B), or C for GAPDH
574 mRNA signals. Data are presented as mean ± SE (n = 4). *, P <0.05 vs. the relevant
575 control.
576

577 **Figure 5. Nrf2 silencing further increased re- UVA irradiation induced cell**
578 **damage; while Nrf2 overexpression reduced re- UVA irradiation induced cell**
579 **damage.** FEK4 cells were transfected with siNrf2 (A and B) or Nrf2 (C) for 48 h, then

580 either sham or UVA-irradiated and re-incubated, prior to a second UVA treatment (as
581 described in Fig. 4). At 8 h following the re-irradiation, membrane damage (A, C) and
582 cell viability (B, D) were determined by the LDH and MTS assays, respectively. The
583 percentage of LDH leakage and viability were expressed as relative fold changes
584 compared with the sham irradiated control, set as 1 (A) and 100 (B), respectively. Data
585 are presented as mean ± SE (n = 4). * $P<0.05$ vs. the relevant control.