RESEARCH ARTICLE

Curcumin induces stress response and hormetically modulates wound healing ability of human skin fibroblasts undergoing ageing in vitro

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Abstract Wound healing becomes impaired in several diseases and during ageing. A commonly used model for the study of wound healing is a scratched monolayer of cells in vitro, which is convenient for the analysis of the cellular and molecular changes occurring during the two phases of wound healing, namely cell migration and cell proliferation. Cell migration, which is the primary event to occur during initial wound healing, is inversely dependent on the number of focal adhesions (FA) that attach cells to the extracellular matrix. Here we report that the number of FA, measured by determining the levels of FA-proteins paxillin and talin, increase with increasing population doubling level of the serially passaged normal adult skin fibroblasts, and that this increase may account for the age-related slowing down of wound healing in vitro. We also report that curcumin, a component of the widely used spice turmeric, modulates wound healing in vitro in a biphasic dose response manner, being stimulatory at low doses (between 1 and 5 μM), and inhibitory at higher doses. Furthermore, our results show that the hormetic effects of low levels of curcumin are achieved by virtue of it being a hormetin in terms of the induction of stress response pathways, including Nrf2 and HO-1 in human cells.

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Introduction

Wound healing in the skin becomes severely limited during ageing and age-related diseases, including diabetes and some cancers. Although at the tissue level, wound healing is a complex phenomenon involving various cells, cytokines and angiogenesis, at the cellular level it is mainly carried out in two steps: cell migration and cell proliferation. Whereas age-related alterations in cell proliferation rate during ageing are well described (Campisi and D'Adda Di Fagagna 2007; Rattan 2010), changes in cellular motility and migration during ageing are yet to be fully elucidated. Cellular migration is highly dependent on the attachment and interaction of a cell with its surroundings, which is facilitated by specific membrane structures termed focal adhesions (FA). FA involve more than ten proteins which connect the intracellular cytoskeleton to the trans-membrane and to the extracellular integrins, which are attached to the extracellular matrix (Mitra et al. 2005). It has been shown that there is a negative correlation between the number of FA and the cellular migration velocity of mouse dermal fibroblasts in vitro (Jurjus et al. 2008). However, there is little such information available for normal human skin fibroblasts (HSF),

especially with respect to ageing and the possibility of modulation by food components.

Serially passaged HSF are widely used as an experimental system for unravelling the cellular, biochemical and molecular mechanisms of ageing and age-related alterations in vitro (Rattan 2010). For studies on wound healing, a frequently used in vitro model is a scratched monolayer of fibroblasts, which is also the basis for testing the effects of natural and synthetic potential modulators of wound healing (Liang et al. 2007). One natural compound claimed to promote wound healing is curcumin—a food component of a widely used spice, turmeric. There is a plethora of biological effects of curcumin reported along with a variety of molecular mechanisms involved (Aggarwal and Harikumar 2009). Most of the studies with curcumin have focused on its anti-cancer, anti-cell proliferative, anti-inflammatory and other inhibitory effects at relatively high concentrations (above 10 μM). There is very little information available on the effects of curcumin on normal HSF undergoing ageing in vitro. Our previous studies have shown that at lower concentrations, curcumin has various stimulatory effects in human skin cells, such as the stimulation of the proteasome, increased differentiation of keratinocytes, and enhanced stress tolerance (Ali and Rattan 2006; Berge et al. 2008; Lima et al. 2010). This biphasic or hormetic nature of the effects of curcumin needs further investigation. Here, we present the results of our studies which show that curcumin hormetically promotes cellular motility, migration and would healing in HSF undergoing ageing in vitro. We also provide evidence that induction of stress response (SR) is the primary molecular mode of action of curcumin in bringing about these effects, thus making it a potential hormetin.

Methods

Cell culture

Normal adult skin fibroblasts (designated ASF-2) were derived from a breast biopsy specimen of a consenting healthy 28-year-old Danish woman, as described earlier (Rattan and Sodagam 2005). Cells were grown in monolayers in plastic tissue culture flasks (NUNC, Roskilde, Denmark), and were incubated under normal culturing conditions of 37°C, 95% relative humidity and 5% CO_2. The complete culture medium was comprised of Dulbecco's modified eagle medium, DMEM (Biowhittaker, Viviers, Belgium) supplemented with 10% bovine fetal calf serum (FCS) (Biological Industries, Beit Haemek, Israel), 2 mM L-glutamine (Biowhittaker) and 100 U/ml penicillin/streptomycin (Biowhittaker). When cultures were near confluent (90–95% confluency), the cells were released from the culture flask surface by first washing with PBS and then harvesting with 0.25% trypsin/EDTA solution. Trypsin treatment was for about 5 min, and its action was inhibited by the addition of complete medium. Cells were counted using an automated electronic cell counter (Coulter Z2, Ramcon A/S, Denmark) to keep track of the population doublings (PD). Cells were serially passaged by usually splitting the near confluent cultures at a 1:4 ratio every week, except for late passage cells (>75% lifespan completed), which were split 1:2 due to the decreased proliferative activity.

Wound healing scratch assay

After trypsinization 200,000 cells were seeded in each well (9 cm²) in a 6-well plastic plate (NUNC, Roskilde, Denmark). The "wound" or scratch was made with a custom-made plastic cell scraper with 2.2 mm width and with sharp edges. The medium containing the scraped cells was replaced by fresh medium with or without stated amounts of curcumin [1,7-bis(4-hydroxy-3-methoxyphenyl)-92   1,6-heptadiene-3,5-dione]; Sigma-Aldrich) dissolved in DMSO (0.25% final concentration in stock solution). The wound was allowed to "heal" for up to 72 h before the cells were fixed in 100% ice-cold methanol for 20 min at −20°C, and stained with Giemsa's methylene blue (Merck KGaA, Germany) for 1 h. The quantification of the wound was made by measuring the scratched area not covered by cells, by custom definition with Zeiss AxioVision v. 4.6.3.0, using the image analysis (as described in Rattan et al. 2007).

Immunocytochemistry and flow cytometry

Cells were seeded in 24-well plates (NUNC, Roskilde, Denmark) at low density (about 5,000 cells per cm²) 24 h prior to the treatment. Cells were fixed with

4% paraformaldehyde and permabilized with 0.2% Triton X-100. Cells were incubated with primary antibody in 2% skim milk and washed before incubating with secondary antibody. The subsequent wash was followed by mounting the cells with the mounting medium (Vectashield, Burlingame, USA) containing DAPI for DNA detection. The antibodies used were as follows: paxillin (H-114 from Santa Cruz Biotechnology, USA), talin (8D4 from Sigma-Aldrich, USA), Nrf2 (H300 from Santa Cruz Biotechnology, USA), HSP70 (SPA810, Stressgen, USA) HO-1 (OSA-110 from Stressgen, USA). Cells used for flow cytometry were harvested by trypsinization, centrifuged at $300 \times g$, and fixed with 70% methanol (4°C) followed by 0.25% paraformaldehyde. After incubation with antibodies, the cells were analyzed immediately with a FACS-Calibur analyzer (Bench flow cytometer), and data was acquired by CellQuest Pro.

Western blotting

Cells were harvested by scraping on ice or by trypsinization and centrifuged at $300 \times g$ for 5 min. Protein quantification was done by using Plus One 2D Quant Kit (Amersham). After adding sample buffer ($4 \times$, BIO-RAD) and XT reducing agent ($20 \times$, BIO-RAD) the sample was boiled for 5 min before loading to the prefabricated 12% Bis–Tris SDS-PAGE gels (BIO-RAD). The gel was transferred to a nitrocellulose membrane (BIO-RAD) for 1.5 h at 100 volts at 5°C. After blocking in 2% skim milk the membrane was incubated with primary antibody for 1 h followed by three washes with 0.05% Tween–PBS and incubation with secondary antibody. The membrane was processed with chemiluminescent ELC (GE-Healthcare) before developing the film (Kodak MedicalFilm) in a dark room with a developer (AGFA Curix 60).

Software data analysis

Western blots were quantified using Adobe Photoshop® CS4 after scanning the developed film, and acquiring the numerical values of the bands relative intensity. Flow cytometry data were analyzed by FlowJo®. Raw data were analyzed according to the cell type and cell size, for application of the correct gates to exclude cell debris. Differences between groups were considered significant when P-values

were less or equal to 0.05, and were analyzed by one-way ANOVA followed by the Student–Newman–Keuls multiple comparisons test, using GraphPad Prism 4.0 software (San Diego, CA, USA).

Results and discussion

Replicative lifespan and wound healing in vitro

In this series of experiments starting from PD 6, serially passaged ASF-2 cells achieved a final cumulative population doubling level (CPDL) of 45 in 170 days, which is considered as 100% lifespan completed (LSC) *(data not shown)*. For comparative analyses, cells below 30% LSC were considered as early-passage young cells; cells up to 80% LSC were considered to be middle-aged; and those above 85% LSC were considered as late-passage senescent cells.

The in vitro wound healing is considered to occur in two steps: (i) migration which occurs within the first 24 h post wounding, and (ii) cell proliferation which continues until the wound area is totally filled out again. Since the wound or scratch should have a size that, under optimal circumstances, allows occurrence of both cell migration and division, in the present series of experiments the wound was made optimal for the microscopic photography settings, with the exact width of the wounds being 0.2 mm, that varied by <2%. Figure 1 shows an example of in vitro wound healing in early passage young ASF-2 cells after 48 h, with markings of the initial wound edge.

Two distinct features were observed during in vitro wound healing. First, the orientation of the cells perpendicular to the wound appear to determine which cells would be the first ones to migrate into the cell-free area (marked with circles in Fig. 1), followed by cells which are aligned parallel to the wound. This will entail local differences in the wound healing success rate, which must be taken into account when quantifying the extent of wound healing. The second notable observation during wound healing was the single cell invasion into the cell-free area, which results in only a certain proportion of the cells that migrate to heal the wound.

It is well known that early passage young cells are thin, spindle shaped, and are arranged in a fingerprint-like phenotype which usually is lost in late passage
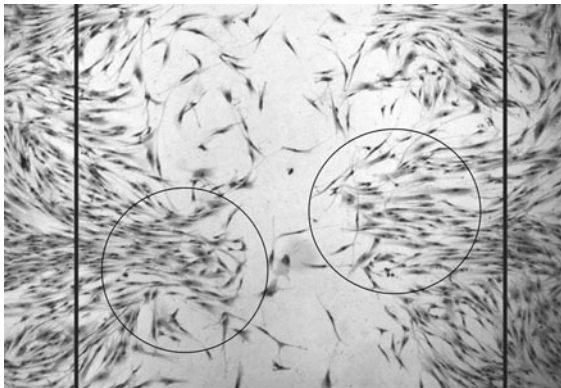
Fig. 1 In vitro wound healing after 48 h in early passage human skin fibroblasts (<40% lifespan completed). The wound closure is highly dependent on cell orientation where cell bridges are formed (indicated by *circles*). The initial wound edge is marked with *black lines*. (Microscopic magnification of the objective lens 10×)

cultures (Rattan 2010). This may contribute towards impaired wound healing during ageing by affecting the number of cells able to initiate migration. With increasing age, there is a further decrease in the number of cells that migrate into the wounded area, which may be due to the enlarged cell size, flattened cell morphology, enhanced cell rigidity and a lack of response to signals which initiate the migration. These possibilities need to be further characterized by single cell-tracking time-lapse microscopy.

Molecular changes in wound healing during ageing

Immunocytochemical fluorescence staining showed an increased level of the two proteins involved in FA, paxillin and talin, with increasing cellular age in vitro. Figure 2 shows examples of early-passage young and late-passage senescent ASF-2 cells stained for talin together with nuclear stain DAPI. The appearance of early-passage young cells was thin and elongated with a distinctly outlined cell membrane, whereas late-passage senescent cells were enlarged, flattened and exhibited an irregular membrane structure. The fluorescent specs in Fig. 2 represent talin which was widespread throughout the cells, and there was a significant difference in its localization between young and senescent cells, resulting in a higher concentration of these proteins on the very
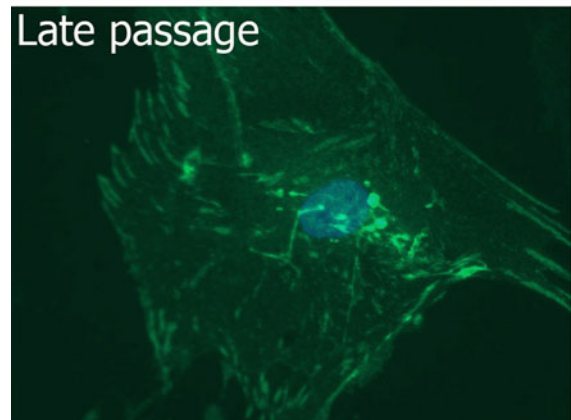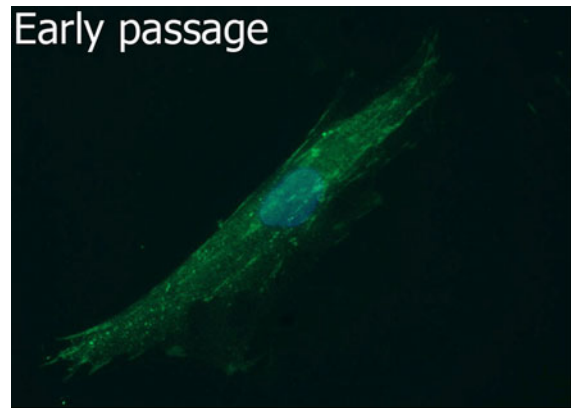

Fig. 2 Immunocytochemical staining of the puncta-forming focal adhesion protein talin in early (<40% lifespan completed) and late passage (>90% lifespan completed) human skin fibroblasts. (Microscopic magnification of the objective lens 40×)

edge of the old cells. Similar observations were made for the other FA-associated protein paxillin, which is adjacent to talin (*pictures not shown*). These results indicate that during serial passaging cells progressively become more rigid and inflexible, which is most likely the reason for their decreased ability to migrate in the wounded area during ageing.

For semi-quantitative comparisons, protein extracts from cells at different PDL in vitro were analyzed by Western blotting, using antibodies against paxillin. Figure 3 shows the Western blot for paxillin with cells at different LSC. By measuring the band intensity of paxillin, a significant increase of approximately 2.5-fold was observed in late passage cells as compared with early passage cells, giving further support to immunofluorescence-microscopy observations. Since Western blots are usually not very
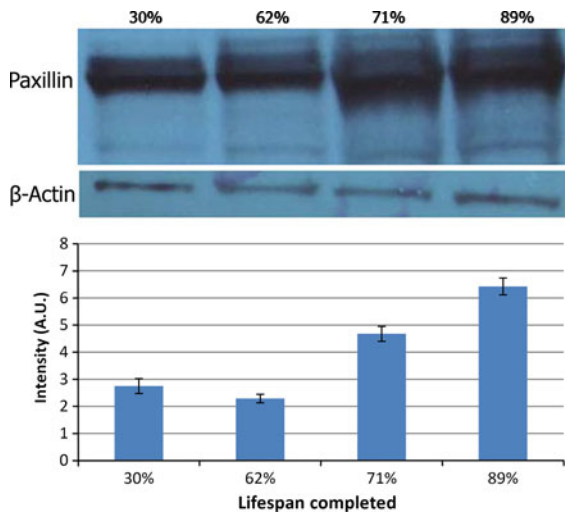
Fig. 3 A representative Western blot and its semiquantitation showing an age-related increase in the amount of paxillin in focal adhesions, in serially passaged human skin fibroblasts undergoing ageing in vitro

precise for quantifying differences below a certain level, further flow cytometric analyses were done. There was a significant increase (about 2.5-fold) in the levels of talin in late passage senescent cells as compared with early passage young cells (Fig. 4a, b). A similar general increase in FA with cellular age in mouse dermal cells has been reported previously, showing a correlation between an increased numbers
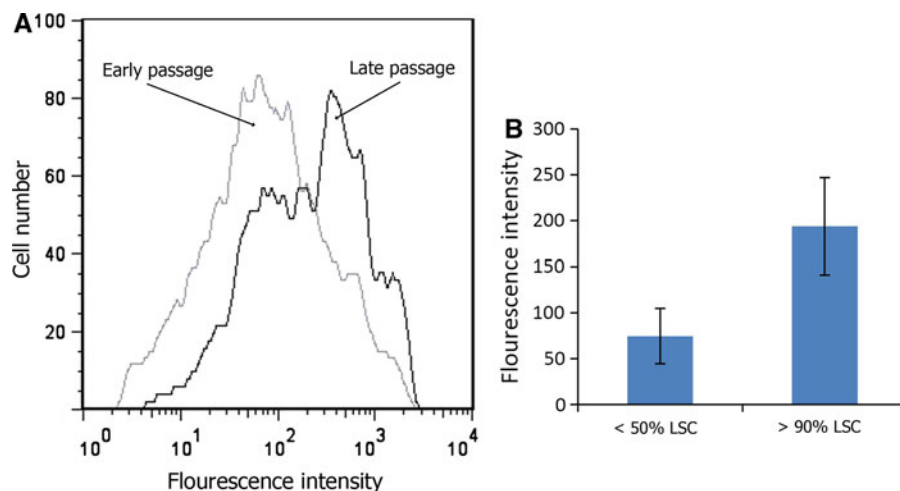
of FA and decreased cellular motility (Jurjus et al. 2008). Thus our results confirm that decrease in motility and decreased wound healing in human cells undergoing ageing in vitro is also due to an increase in the number of FA.

Modulating wound healing with curcumin

We have observed that curcumin has a wound healing promoting effect at low doses, whereas at higher doses it is inhibitory and toxic for cells. Figure 5 shows Giemsa-stained microphotographs of the extent of wound healing in vitro in early passage young HSF treated with different doses of curcumin (0, 0.25, 0.50, 1, 5 and 20 μM) for 24 h. By measuring the cell-free area in pictures shown in Fig. 5, it was possible to determine the extent of the wound closure. Figure 6 shows a biphasic dose–response of curcumin on wound healing, where lower doses were stimulatory while higher doses were inhibitory. Whereas the effect of curcumin on early passage young cells was stimulatory in the very low dose range (about 0.25 μM) and was inhibitory at the doses higher than 5 μM, late passage cells were less responsive to the stimulatory effects of curcumin at 0.25 μM, but were relatively more tolerant to higher doses of up to 5 μM. However, HSF at all ages became inhibited with respect to both proliferation

Fig. 4 Flow cytometric analysis of talin-stained human skin fibroblasts during ageing in vitro. **a** Fluorescence distribution curves: the *grey line* represents early passage (<50% lifespan completed) cells whereas the *black line* shows the distribution of talin in late passage cells (>90% lifespan completed); **b** The columns show the geometrical mean values, with *error bars* representing the area of the histograms
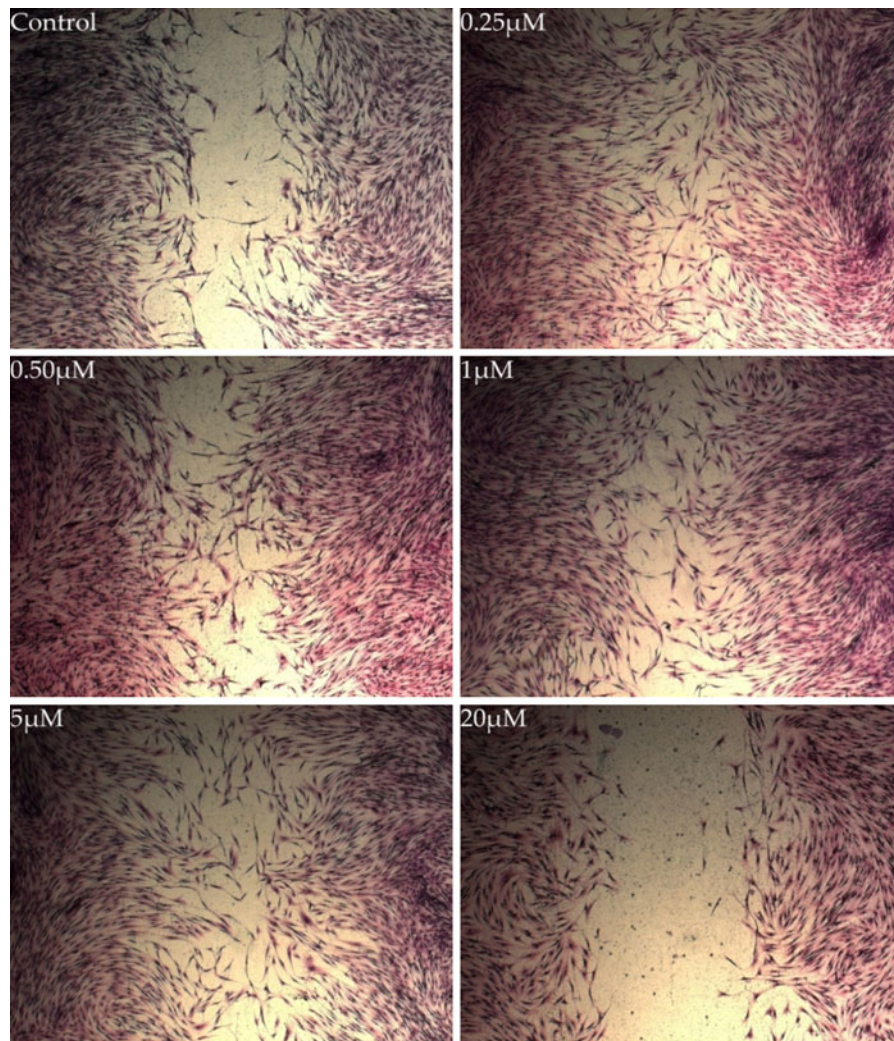
Fig. 5 Effect of various doses of curcumin on in vitro wound healing in early passage human skin fibroblasts (<30% lifespan completed; microscopic magnification of the objective lens 10×)

and migration at doses of 20 μM and above, eventually resulting in apoptotic cell death *(not shown)*. For further elucidation of the age-related differences in the effects of curcumin on wound healing in vitro, it is important to study the effects of curcumin on the extent of FA formation throughout the replicative lifespan of serially passaged cells.

Curcumin as a hormetin

Although a large number of eventual biological effects of curcumin are well documented, very little is known about the immediate and early events initiated by curcumin within the cells. There are

indications that curcumin is a proton-donor and is not a direct effector molecule in terms of being a strong free radical scavenger (Jovanovic et al. 1999). Rather, the presence of curcumin inside or outside a cell may be interpreted as a disturbance or stress for the cellular homeodynamics, which initiates one or more stress response (SR) pathways in order to re-establish homeodynamics. It has been suggested that curcumin is involved in the disruption of Nrf2–Keap1 complex by electrophilic modification of Cys-151 in Keap1 (for details, see Singh et al. 2010). Activation and translocation of Nrf2 to the nucleus leads to its binding to antioxidant response element (ARE), followed by the upregulation of several antioxidant
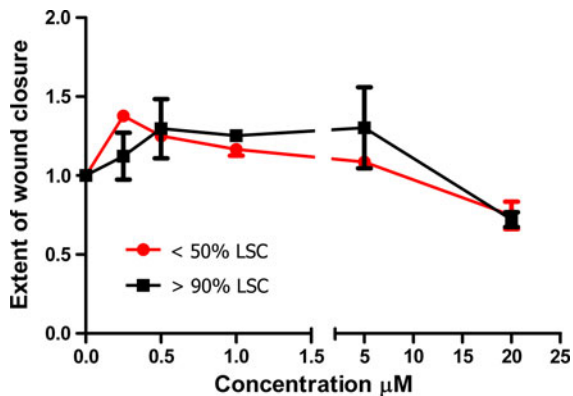
Fig. 6 Biphasic dose response of the effects of curcumin on the extent of wound healing in early- (<50% lifespan completed) and late-passage (>90% lifespan completed) human skin fibroblasts

genes. Such conditions of mild stress, which initiate SR and can result in achieving biologically beneficial effects are termed as hormetins (Ali and Rattan 2006; Rattan 2008). There is some evidence that curcumin acts as a mild stress-inducing hormetin via Nrf2–ARE stress response (Lima et al. 2010; Calabrese et al. 2008; Singh et al. 2010).

Figure 7 shows the effects of curcumin on the induction of different stress markers in early passage young HSF. We have used a 1 h HS at 41°C as a reference stressor, which is known to induce the synthesis of a primordial stress protein, Hsp70 (also known as HSPA1A). The main SR initiated by curcumin appears to be the oxidative damage-induced activation of the transcription factor Nrf2 (Fig. 7). ASF-2 cells treated with 5 μM curcumin for 24 h had a fivefold increase in the level of Nrf2 as detected by Western blotting. A combination of curcumin and HS also induced Nrf2 response, but to a lower extent (about threefold). HS on its own also induced Nrf2 response (twofold) as compared with the controls. Since the initial phase of the Nrf2 response is the translocation of Nrf2 to the nucleus, it will be important to confirm the translocation of Nrf2 in the presence of curcumin.

In the case of a downstream effector of Nrf2, haemeoxygenase-1 (HO-1), also known as Hsp32, curcumin on its own or in combination with HS increased the levels of HO-1 significantly in HSF (Fig. 7). But in the case of Hsp70, curcumin on its own had no effect on the induction of this stress protein, but it acted as a co-inducer when cells were
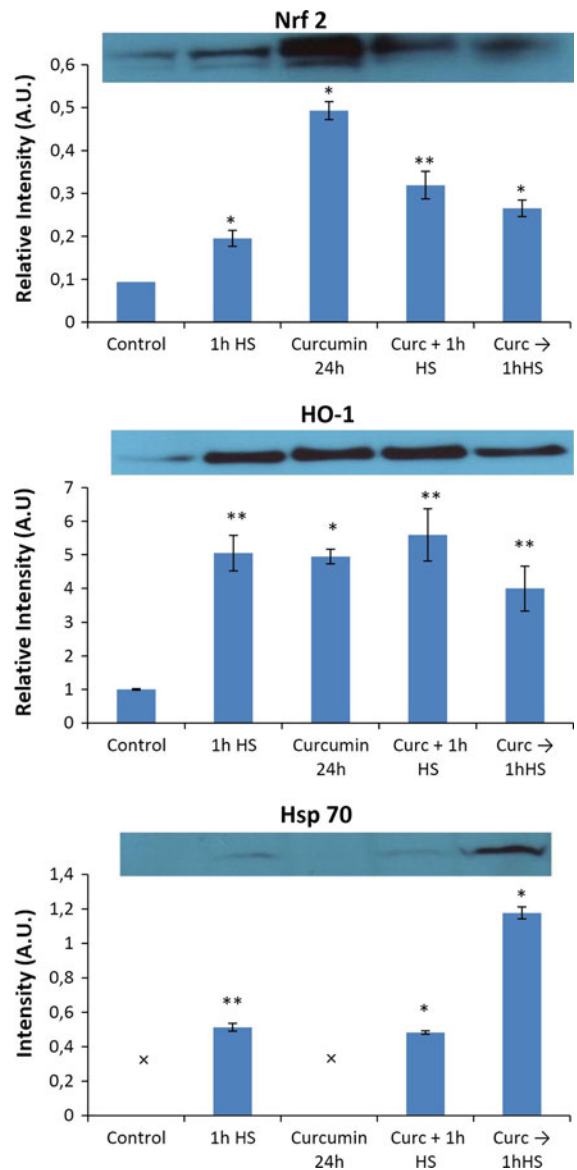

Fig. 7 Representative Western blots and semiquantification of the effect of curcumin and mild heat shock (41°C) on the levels of stress markers Nrf2, HO-1 and Hsp70 in early passage (<50% lifespan completed) human skin fibroblasts. *P < 0.01, **P < 0.05

pretreated with curcumin for 24 h before giving 1 h HS at 41°C. However, a post-treatment of cells with curcumin after HS did not induce any additional synthesis of Hsp70. This confirms the previous report that curcumin acts as a co-inducer of Hsp70 (Rattan et al. 2007). Any differences between early-passage young cells and late-passage old cells with respect to

the extent of various stress responses in the presence of curcumin are yet to be mapped out.

Activation and induction of Nrf2 and HO-1 indicate that curcumin does not act directly as a chemical anti-oxidant by quenching free radicals, but may be a mild pro-oxidant, which triggers the cells' own anti-oxidant systems to remove oxidative damage, thus bringing about its anti-oxidative end-results. However, the exact nature and extent of the oxidative damage induced by curcumin, which leads to the activation of the Nrf2 stress pathway followed by a downstream activation of numerous anti-oxidative genes, is yet to be elucidated. Numerous targets of curcumin have been reported, including SP-1, p53, STAT-3, ATF3, Nrf2, PPAR-γ, CHOP, NFκB, iNOS and HIF-1, justifying its eventual antioxidant, chemopreventive and anti-inflammatory activities (see, Calabrese et al. 2010). Our studies suggest that curcumin hormetically brings about its biological effects by virtue of it being a hormetin in causing some molecular damage, which then induces cellular stress responses as a defense mechanism.

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