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Curcumin induces heme oxygenase-1 in normal human skin fibroblasts through redox signalling: relevance for anti-aging intervention

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Title: Curcumin induces heme oxygenase-1 in normal human skin fibroblasts through redox signalling: relevance for anti-aging intervention

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List of Abbreviations: ARE: Antioxidant Response Element; GST: glutathione-*S*-transferase; H_2DCFDA: 2',7'-Dichlorodihydrofluorescein diacetate; HO-1: heme oxygenase-1; Hsp: heat shock protein; Keap1: Kelch-like ECH-associated protein 1; MARS: maintenance and repair systems; NAC: N-acetyl cystein; Nrf2: nuclear factor-erythroid-2-related factor 2; ROS: reactive oxygen species; *tert*-BOOH: *tert*-butyl hydroperoxyde.

Keywords: anti-aging, antioxidant defences; curcumin; normal human skin fibroblasts; hormetic effects.

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Abstract:

Curcumin, a component of the spice turmeric, was tested for its potential hormetic anti-aging effects as an inducer of mild stress. Early passage young human skin fibroblasts treated with low doses of curcumin (below 20 μM) showed a time- and concentration-dependent induction of heme oxygenase-1 (HO-1), followed by compensatory increase in GST activity, GSH levels and GSH/GSSG ratio. These effects were preceded by induction of oxidative stress (increased levels of ROS and DNA damage) and impairment of cells' redox state. Curcumin also induced Nrf2 accumulation in the nuclei. The use of the antioxidant NAC prevented the induction of HO-1 by curcumin. Pharmacological inhibition of PI3K, but not other kinases, significantly prevented curcumin-induced HO-1 levels, which was corroborated by the induction of phospho-Akt levels by curcumin. Late passage senescent cells already had higher HO-1 levels, and further induction of HO-1 by curcumin was considerably impaired. The induction of stress responses by curcumin in human cells led to protective hormetic effects to further oxidant challenge. In conclusion, curcumin induces cellular stress responses in normal human skin fibroblasts through PI3K/Akt pathway and redox signalling, supporting the view that curcumin-induced hormetic stimulation of cellular antioxidant defenses can be a useful approach towards aging intervention.

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1 Introduction

Several dietary factors have been linked to health and longevity. Fruits and vegetables are among the dietary components for which epidemiologic studies suggest an association between their consumption and a decreased risk of premature death and mortality from certain age-related diseases [1]. Many of the phytochemicals present in plants are generally accepted as contributors towards these health positive effects. Besides other intrinsic properties of these compounds, such as being direct antioxidants, phytochemicals may also be beneficial by working as hormetins by mild stress-induced induction of pathways of protection, maintenance and repair [2, 3]. Curcumin is one such phytochemical for which several studies already exist relating its health beneficial effects, mainly as an anti-inflammatory and anti-cancer compound at relatively high doses [4]. However, little is known about the effects and molecular mechanisms of action of curcumin on normal human cells undergoing aging.

Biological aging is a fundamental process where the progressive decline of organismal fitness and cellular functions represent major risk factors for the development of age-related diseases and eventual death. Substantial data support the view that the accumulation of intra- and extra-cellular molecular damage during the aging process results in part from the progressive failure of cellular maintenance and repair systems (MARS) [5]. Therefore, stimulating MARS has been increasingly recognized as an important approach for aging intervention and prevention [5, 6]. Mild stress-induced stimulation of MARS represents a promising strategy to achieve this goal. Such adaptive responses of biological systems to a low or intermittent dose of otherwise harmful condition resulting in protection against subsequent stresses is known as hormesis [2, 7]. Some potential hormetic agents (hormetins), such as

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physical (radiation, temperature and exercise), chemical (hormones and pro-oxidants) and nutritional (food restriction and nutritional components) hormetins, have been tested and shown to bestow beneficial and anti-aging effects on various aging systems, including human cells [3, 8]. We have recently reported that curcumin could be a hormetin since it was able to significantly increase heme oxygenease (HO-1) expression, one cytoprotective enzyme under the influence of oxidative stress response, in normal human skin fibroblasts [9].

Curcumin (diferuloylmethane) is the active polyphenolic compound present in the rhizome of the Indian spice *Curcuma longa* Linn (turmeric, Zingiberaceae family), and it has been receiving increasing attention due to its ability to induce cellular stress responses as well as repair, detoxifying and antioxidant pathways. For example, curcumin was able to co-induce heat shock proteins (Hsps), in particular Hsp70, in rat cells when co-treated with various stresses, including heat shock [10]. Recently, curcumin alone was reported to induce the heat shock response in human leukemia cells, in particular causing the nuclear translocation of the heat shock transcription factor and increasing the expression of Hsp70 at transcriptional and translational levels [11]. From a biphasic dose response hormetic view, curcumin at low concentrations was able to stimulate proteasome activity, but was inhibitory at high concentrations [12]. Several studies also report the ability of curcumin to induce protective proteins such as HO-1, phase II enzymes and glutamate cysteine ligase under the Antioxidant Response Element (ARE) [13-19]. Some of these studies showed that the induction of these enzymes conferred further protection against oxidative stress in endothelial [14], neuronal [13] and vascular smooth muscle [16] cells, corroborating curcumin's hormetic effects.

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Several reports have shown that the induction of many of these detoxifying and antioxidant cytoprotective enzymes by curcumin are mediated by the transcription factor nuclear factor-erythroid-2-related factor 2 (Nrf2) through activation of the cis-acting element ARE [16, 20]. Also *in vivo*, oral administration of curcumin resulted in the induction of Nrf2-dependent genes in the liver and small intestine of rodents [21-23]. In unstressed cells, Nrf2 controls basal expression of its target genes and is constantly targeted by the Kelch-like ECH-associated protein 1 (Keap1) for degradation catalyzed by the 26 S proteasome via the ubiquitin-dependent pathway [24]. Reactive chemical stresses or other ARE-inducers lead to Nrf2 accumulation in the nucleus that results in increasing transcription of many cytoprotective enzymes. Many studies showed that curcumin is able to stabilize Nrf2 leading to its accumulation in the nucleus. Inactivation of Keap1 through modifications of its cysteine thiols, and/or phosphorylation of serine or threonine residues of Nrf2 by different kinases has been suggested as possible molecular mechanisms behind Nrf2 stabilization by curcumin [25].

Therefore, the fact that the induction of HO-1 by curcumin is indicative of its potential hormetic ability, we have undertaken studies to elucidate the effects of curcumin in normal human skin fibroblasts undergoing aging *in vitro*. Normal diploid human cells that undergo progressive aging during serial passaging *in vitro* and eventually become replicatively senescent have been used extensively to study the molecular basis of cellular aging [8]. In addition, this same model has been used to test the hormetic effects of different stressors at the level of various cellular functions [8].

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2 Materials and Methods

2.1 Chemicals and antibodies

Curcumin (1,7-bis[4-hydroxy-3-methoxyphenyl]-1,6-heptadiene- 3,5-dione), DMEM, antibiotic/antimycotic solution, kinase inhibitors PD98059, SB203580, SP600125, LY294002, RO320432 and staurosporine, *tert*-butyl hydroperoxyde (*tert*-BOOH), anti-β-actin antibody as well as all other reagents were from Sigma-Aldrich (St. Louis, MO, USA), unless otherwise specified. Fetal bovine serum (FBS) was bought from Lonza (Verviers, Belgium). 2',7'-Dichlorodihydrofluorescein diacetate (H_2DCFDA) was purchased from Molecular Probes (Eugene, OR, USA). Antibodies against HO-1, Hsp27, Hsp70 and Hsp90 were obtained from Stressgen (Victoria, British Columbia, Canada). Anti-Nrf2, anti-phospho-ERK1/2, anti-JNK, anti-phospho-JNK and anti-p38 antibodies was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies against phospho-Akt (Ser473), total Akt, p44/42 MAPK (ERK1/2) and phospho-p38 MAPK (Thr180/Tyr182) were purchased from Cell Signaling (Danvers, MA, USA). Secondary antibodies HRP donkey anti-rabbit and sheep anti-mouse were purchased from GE Healthcare (Bucks, UK).

2.2 Cell culture

Normal diploid adult human skin fibroblasts (ASF-2 cells) isolated from a breast biopsy specimen of a consenting young healthy Danish woman (aged 28 years) [26] were used. ASF-2 cells were maintained at 37ºC in a humidified 5% CO2 atmosphere grown in DMEM supplemented with 10% FBS, 10 mM Hepes and 1% antibiotic/antimycotic solution. Cell cultures were split in the ratio 1:2 or 1:4 after

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they became confluent. All experiments were performed with ASF-2 cells with cumulative populations doublings between 15 and 25 (25-45% of replicative lifespan completed) at a density of 100,000 cells per well in a 6-well plate, unless otherwise specified. Some experiments were also performed with late passage senescent ASF-2 cells with cumulative populations doublings around 45 (~90% of replicative lifespan completed) at a density of 50,000 cells per well in a 6-well plate. All experiments were performed 24 h after seeding using complete medium. Curcumin and kinase inhibitors were added to culture medium dissolved in DMSO (final concentration in the assays were 0.5% (v/v)); controls received vehicle only.

2.3 Cell viability and growth

Necrosis induced by curcumin to human skin fibroblasts was evaluated visually under a phase contrast light microscope by severe morphological changes with appearance of blebs in the cytoplasmic membrane.

The effects of curcumin on cell viability and growth was evaluated by the MTT assay in 96-well culture plates as previously described [27] with some modifications. Briefly, 2 h before the end of the treatment period, cells were incubated with MTT to a final concentration of 0.5 mg/ml. Then, the medium was removed, and the formazan crystals formed by the cell's capacity to reduce MTT were dissolved with a 50:50 (v/v) DMSO:ethanol solution, and absorbance measured at 570 nm (with background subtraction at 690 nm). The results were expressed as percentage relative to the control (cells without any test compound). MTT reduction at the beginning of incubation period (t = 0 h) was performed and represented in the graph as a line. Since the effects of the compounds were studied after 72 h of incubation and cells grew

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significantly within this time period, the point at the beginning of the incubation (line in the graph) allows to distinguish between cell death (values below line) and inhibition of proliferation (values between line and 100%).

In the experiment where the hormetic potential of curcumin was tested, ASF-2 cells were incubated for 2 h with 10 μM curcumin, and then the medium was removed and replaced with fresh medium. After 16 h of recovery, cells were incubated with 200 μM *tert*-BOOH for 3 h and cell viability measured by the LDH leakage method as previously described [28].

2.4 ROS levels

The levels of reactive oxygen species (ROS) in ASF-2 cells treated with curcumin were measured using the H_2DCFDA probe following manufacturer's instructions. H_2DCFDA in the presence of ROS is oxidized to highly fluorescent DCF and can be monitored by fluorometry. In brief, 12,000 cells/well were seeded in a cell culture-treated black 96-well plate. One day after seeding, cells were washed with HBSS and then loaded with 10 μM H_2DCFDA (or vehicle for controls) for 30 min. Afterwards, cells were washed again with HBSS and incubated with different concentrations of curcumin for 30 min (100 μM *tert*-BOOH was used as positive control). Then, after washing cells with HBSS, the fluorescence of DCF was detected in a microplate reader fluorometer POLARstar OPTIMA (BMG Labtech, Offenburg, Germany) and the results expressed as the DCF fluorescence increase relative to the control situation.

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2.5 Comet assay

The potential of curcumin to induce DNA damage to ASF-2 cells was evaluated by the alkaline version of the comet assay as previously described [29]. Briefly, after treating cells for 1 h with different concentrations of curcumin (100 μM *tert*-BOOH was used as a positive control), cells were resuspended using trypsin solution, embedded in 0.5% (w/v) low melting agarose and spread onto agarose coated slides. Cells were then lysed to expose DNA, and the nucleoids electrophoresed for 20 min at 0.8 V/cm, 300 mA, in a cold room (4 °C). After neutralization, comet images were analyzed by visual scoring under a fluorescent microscope and 100 nucleoids per slide counted.

2.6 GSH content

Protein from cell scrapes were pelleted in 5% (w/v) 5-sulfosalicilic acid and the glutathione levels determined in the supernatant by the DTNB–GSSG reductase recycling assay as previously described [30]. The results were expressed as nmol GSH/mg of protein.

2.7 GST activity

For measurement of glutathione-*S*-transferase (GST) activity, cell homogenates were centrifuged at 10,000 ×*g* for 10 min at 4 °C and the supernatant collected. The activity of GST were measured spectrophotometrically at 37 °C as previously described [28], and the results expressed as percentage from control.

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2.8 Protein extraction and western blotting

To determine the level of proteins of interest, ASF-2 cells were treated with different concentrations of curcumin for the indicated times, cell monolayers were washed twice with PBS and then lysed in ice cold lysis buffer (50 mM Tris-HCl, pH 8, 150 mM NaCl, 0.5% (v/v) NP-40, 0.1% (v/v) SDS, 0.1% (v/v) deoxycholate, 1 mM EDTA, 1 mM EGTA) containing 1 mM PMSF, 1 × complete protease inhibitor cocktail (Roche, Mannheim, Germany) and phosphatases inhibitors (20 mM NaF, 20 mM $Na_2V_3O_4$). When kinase inhibitors, NAC and other antioxidants were used, they were pre-incubated for 30 min before addition of curcumin.

For preparation of nuclear extracts, cells were washed twice with PBS and nuclear fractions prepared as described elsewhere [13]. In brief, cells were incubated with ice cold hypotonic buffer (10 mM Hepes, pH 7.9, 10 mM KCl, 1.5 mM $MgCl_2$) containing 0.5 mM DTT, 1 mM PMSF and 1 × complete protease inhibitor cocktail (Roche) for 15 min to allow cell swelling. Then, 10% (v/v) NP-40 were added to a final concentration 0.7% (v/v) and the microtube vortexed for 15 sec vigorously. The homogenate was centrifuged at 10,000 ×*g* for 5 min at 4ºC, cytosolic supernatant harvested and the nuclear pellet was resuspended in ice cold nuclear buffer (20 mM Hepes, pH 7.9, 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA) containing 0.5 mM DTT, 1 mM PMSF and 1 × complete protease inhibitor cocktail. The nuclear fraction was then incubated on ice for 15 min and vortexed for 15 sec each 2 min. Then, the nuclear extract was centrifuged at 18,000 ×*g* for 5 min at 4ºC and the supernatant containing the nuclear proteins harvested.

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Protein concentration was quantified using the Bio-Rad DC protein assay (Bio-Rad Laboratories, Inc., Hercules, CA, USA) and BSA used as protein standard.

For western blot, 20 µg of protein were resolved in SDS-polyacrylamide gel and then electroblotted to a Hybond-P polyvinylidene difluoride membrane (GE Healthcare). Membranes were blocked in TPBS (PBS with 0.05% Tween-20) containing 5% (w/v) non-fat dry milk, washed in TPBS and then incubated with primary antibody. After washing, membranes were incubated with secondary antibody conjugated with IgG horseradish peroxidase and immunoreactive bands were detected using the Immobilon solutions (Millipore, Billerica, MA, USA) under a chemiluminescence detection system, the Chemi Doc XRS (Bio-Rad Laboratories, Inc.). Band area intensity was quantified using the Quantity One software from Bio-Rad. β-actin was used as loading control.

2.9 Immunofluorescence

ASF-2 cells were treated with curcumin 10 µM for the indicated time, washed with HBSS and then fixed with 4% paraformaldehyde in PBS (pH 7.4) at room temperature for 5 min. After washing cells three times with PBS, they were permeabilized with 0.2% Triton X-100 in PBS for 5 min, washed two more times with PBS, and blocked with 1% BSA in TPBS for 30 min. Cells were then incubated with mouse anti-HO-1 antibody (1:500) for 1 h at room temperature in a humid atmosphere. After cell washing, incubation with secondary anti-mouse IgG antibody conjugated with FITC (Dako, Glostrup, Denmark) was performed for 45 min at room temperature in a dark humid atmosphere. The cells were then washed two times with TPBS, mounted with VECTASHIELD containing DAPI (Vector Laboratories,

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Burlingame, CA, USA) and observed under a confocal microscope (LSM 510 META, Zeiss, Gottingen, Germany).

2.10 Statistical analysis

Data were presented as mean ± SEM. Statistical differences among data groups were analyzed by one-way ANOVA followed by the Student-Newman-Keuls multiple comparison test, or analyzed by the Student's *t*-test when appropriate, using GraphPad Prism 4.0 software (San Diego, CA, USA). Differences between groups were considered significant when P-values ≤ 0.05.

3 Results

3.1 Curcumin Affects Cell Viability and Growth

Fig. 1 shows that exposure of normal adult human skin fibroblasts ASF-2 to 5-20 μM curcumin for up to 3 days inhibited cell proliferation in a concentration dependent manner, as determined by the cell's capacity to reduce MTT (Fig. 1 A), and by counting the cell numbers (Fig. 1 B). The extent of cell growth inhibition by curcumin was statistically significant only at 10 μM (36% in the MTT assay and 65% in cell counting) and above (~87% inhibition at 20 μM). Treatment with 30 μM curcumin for 24 h resulted in significant cell death by necrosis as evident from severe morphological changes and the appearance of blebs in the cytoplasmic membranes (data not shown).

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3.2 Curcumin Induces HO-1 Levels in Human Skin Fibroblasts

The effects of curcumin on the expression of different Hsps and HO-1 in normal diploid human skin fibroblasts were studied by western blot. As shown in Fig. 2 A, treatment with 10 µM curcumin for 24 h remarkably induced the expression of HO-1 in ASF-2 cells, but did not change significantly the expression of Hsp27, Hsp70 and Hsp90. Curcumin was also unable to co-induce the expression of these Hsps after a heat shock treatment of 1 h at 41ºC (Fig. 2 A). On the other hand, curcumin induced in a concentration- and time-dependent manner the expression of HO-1 in ASF-2 cells (Fig. 2 B & C).

3.3 Curcumin Increases ROS and Induces DNA Damage

In view of the induction of HO-1 stress protein by curcumin, we next asked whether this would be the result of oxidative stress imposed on the cells. Incubation of ASF-2 cells with curcumin for 30 min resulted in a concentration-dependent significant increase in ROS levels, as measured by the DCF probe (Fig. 3 A). Curcumin (10 µM)-induced ROS levels were significantly decreased (about 17%) when cells were co-incubated with the antioxidant NAC. In addition, using the alkaline version of the comet assay, curcumin showed induction of DNA damage in a concentration-dependent manner after 1 h of incubation (Fig. 3 B). When incubated with 20 µM curcumin, almost 100% cells showed mild or moderate DNA damage (Fig. 3 C). Therefore, in the short term curcumin seems to induce oxidative stress to the cells, which may explain the further induction of HO-1.

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3.4 Oxidative Stress is Involved in Curcumin-Induced HO-1 Expression

To explore whether oxidative stress is involved in the expression of HO-1 induced by curcumin, a co-incubation with the well-known antioxidant NAC was performed. As shown in Fig. 4, NAC remarkably prevented the curcumin-induced increase of HO-1 (by about 60-80%), as shown both by western blot and immunofluorescence. However, other antioxidants of reference, such as trolox (a water-soluble vitamin E derivative) and α-tocopherol (an absorbable vitamin E form), were unable to inhibit the induction of HO-1 by curcumin. This indicates that NAC's capacity to restore cell redox state may be the basis of its preventive effect, rather than its direct ROS-scavenging ability.

3.5 Cells' Redox State and Signalling are Modulated by Curcumin

To determine if curcumin affects the redox state of ASF-2 cells, the glutathione levels in cells treated with curcumin were determined. As shown in Fig. 5 A, after 1 h of incubation with 10 µM curcumin, a significant 10% decrease in GSH levels was observed, which continued to decrease until 4 h of incubation. However, after 16 h and 24 h of curcumin incubation, GSH levels increased to more than double (Fig. 5 A). GSSG accompanied this increase (Fig. 5 B) but to a lower extent, which resulted after 24 h of incubation with a more than 50% increase in the GSH/GSSG ratio (Fig. 5 C), the most common redox couple used to estimate the cells' redox state. Due to the decrease of GSH levels in the first hours of curcumin incubation, GSH/GSSG ratio also progressively decreased until 4 h before the significant increase by the end of incubation period. This indicates that curcumin affects negatively the cells' redox state at first, and then cells respond by increasing glutathione synthesis to restore and

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increase the cells' redox state, a typical biphasic response observed in adaptive stress responses. The impairment of redox state in the first hours of curcumin incubation may influence redox signalling. In fact, the enzyme glutamate cysteine ligase involved in GSH synthesis, HO-1 and GST (a phase II enzyme, whose activity was also significantly increased by curcumin treatment in a concentration-dependent manner - Fig. 5 D) are all part of a set of protective enzymes that are under the influence of a redox-sensitive transcription factor – Nrf2 [25, 31]. The nuclear levels of Nrf2 was measured by western blot, and as shown in Fig. 5 E, curcumin induced a remarkable increase of this transcription factor in the nuclear fraction after 2 h and 8 h of incubation. Therefore, the decrease in the redox state by curcumin in the first hours of incubation seems to be involved in the increase of HO-1 expression and GST activity as well as in the increase of GSH content.

3.6 PI3K/Ak Pathway is Involved in Curcumin-Induced HO-1 Expression

Many kinases such as PI3K, PKC, JNK, ERK and p38 may interfere with the phosphorylation of Nrf2 that is assumed to facilitate its accumulation in the nucleus to stimulate gene expression [25, 32]. ASF-2 cells were, therefore, incubated with curcumin in the presence of different kinase inhibitors for 8 h and the expression of HO-1 measured by western blot. As shown in Fig. 6 A, only the PI3K inhibitor LY decreased significantly the expression of HO-1 induced by curcumin. The inhibitors of MAPKK (in MAPK/ERK pathway), JNK and p38 MAPK (PD, SP and SB, respectively) did not modify the effect of curcumin on HO-1 levels. The same happened with RO, an inhibitor of PKC. Staurosporine was also used to inhibit PKC, and a decrease in HO-1 expression was observed (Fig. 6 A), but associated with cell

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death. To confirm the involvement of PI3K/Akt pathway in the induction of HO-1 by curcumin, the expression of phosphorylated (active) form of Akt was measured. Contrary to what had happened with phospho (p)-ERK, p-JNK and p-p38 where no effect was observed, cells incubated with curcumin (for 8 h) increased the expression of p-Akt (Fig. 6 B) corroborating the previous results.

To explore if there was some dependence on the duration of curcumin exposure on p-Akt levels, incubations in the presence of either LY or NAC for 2 and 8 h was performed. As shown in Fig. 6 C, the expression of p-Akt was remarkably higher after 2 h of incubation than after 8 h of incubation, even in the absence of curcumin. As expected LY decreased significantly p-Akt levels. NAC did not affect p-Akt levels after 2 h of curcumin incubation, but surprisingly decreased it after 8 h (Fig. 6 C). Considering the effect of time on p-Akt levels, its expression along the time was determined in the absence and presence of curcumin. As shown in Fig. 6 D, when medium was replaced by fresh medium containing curcumin or vehicle, p-Akt levels increased remarkably in the first hour and then began to decrease continuously in the following hours. The p-Akt level attained after 1 h was higher in the presence of curcumin, and the subsequent decrease on p-Akt levels was slowed down when curcumin was present (Fig. 6 D).

Since an inhibition of PI3K/Akt pathway decreases HO-1 induction by curcumin and changing cells' medium induces this pathway (observed by the increase of p-Akt levels), we next asked if the effects of curcumin on HO-1 expression was dependent on medium change. For that, curcumin was added to the cells in the same medium they were in previously, and the expression of HO-1 and p-Akt measured. As observed in Fig. 6 E (left panel), curcumin added to cells without medium change also induced HO-1, although to a smaller extent than with medium change. This happened

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without an increase in p-Akt at 1 h (data not shown) and 2 h of curcumin incubation (Fig. 6 E); only at 8 h of incubation a slight increase of p-Akt was observed. To determine the medium component that is responsible for the initial increase of p-Akt after medium change, medium of ASF-2 cells were replaced by fresh complete culture medium, by culture medium without serum or by Krebs buffer containing 25 mM glucose. As shown in Fig. 6 E (right panel), glucose and other components of the culture medium are not involved in the activation of PI3K/Akt pathway in ASF-2 cells, which happens only in the presence of serum.

3.7 Curcumin Increases Stress Tolerance in ASF-2 cells

Considering the stress response elicited by curcumin in ASF-2 cells, we next tested whether this effect would condition cells for a following oxidant challenge. For that, cells were incubated with curcumin for 2 h, followed by a recovery period of 16 h where medium was removed and replaced by fresh one, and then exposed to the oxidant *tert*-BOOH. This pulse incubation with curcumin induced significantly HO-1 expression with maximal levels attained 4 h after the beginning of the recovery period, but decreasing after 16 h (Fig. 7 A). The pulse treatment also induced significantly GSH levels after the 16 h recovery period, without significant changes in GSH/GSSG ratio and GSSG levels (Fig. 7 B). As shown in Fig. 7 C, the stress response induced by curcumin incubation for 2 h afforded 16 h later a significant protection against *tert*-BOOH-induced cell death, dependent on curcumin concentration, demonstrating its hormetic effects via a further oxidant challenge.

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3.8 Effects of Curcumin on Senescent ASF-2 cells

To test if curcumin also induces HO-1 expression in late passage senescent cells, ASF-2 cells with ~90% replicative lifespan completed were used. As shown in Fig. 8, senescent cells had already high expression levels of HO-1 in the control condition. Curcumin induced HO-1 expression in a concentration and time-dependent manner in senescent cells (Fig. 8 B & C, respectively) but to a smaller extent than in young cells (Fig. 8 A), which possess a higher proliferating rate. Medium change at the time of curcumin addition to the cells also induced the expression of p-Akt. Curcumin extended the high levels of p-Akt for a longer time (until 8 h; Fig. 8 C), also to a higher extent than that observed in young cells. As in young cells, NAC and LY inhibited the curcumin-induced HO-1 expression (Fig. 8 D). LY inhibited p-Akt levels from the beginning of the incubation, and NAC did the same but only after 8 h of incubation. Interestingly, LY in the presence of curcumin decreased the HO-1 expression levels below the control values after 24 h of incubation (Fig. 8 D).

4 Discussion

Phytochemicals, such as phenolic compounds, are considered to contribute to the positive health benefits of fruits, vegetables and spice consumption [1, 33], and are proposed to be a good source of hormetic compounds, termed hormetins [34]. Here, we have tested the effects of curcumin in normal diploid human skin fibroblasts undergoing aging *in vitro*. We showed that curcumin worked as an hormetin by inducing mild oxidative stress as shown by increased levels of ROS, DNA damage and decrease of GSH content followed by enhanced antioxidant defences, such as HO-1 levels, GST activity, GSH levels and GSH/GSSG ratio. The use of the

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antioxidant NAC prevented the induction of HO-1 by curcumin. This, together with the decrease of GSH content and increased accumulation of Nrf2 in nuclei in the first hours, suggests that a cellular redox state disturbance causes the induction of a stress response by curcumin, rather than a sole increase of ROS levels. The stress response elicited by curcumin in human skin fibroblasts led to hormetic effects via a further oxidant challenge with *tert*-BOOH, which can be beneficial in the process of aging where the cell's own pathways of protection, removal, maintenance and repair are impaired. In fact, an ever-increasing body of evidence shows that low doses of stressors (mild stress) at young age can increase longevity and delay aging or protect from severe stress at old age [3, 35]. As individuals age, there is a gradual decline of cellular antioxidant defences and a reduced ability of these systems to be induced by different stimuli [36]. The decreased ability of senescent fibroblasts to induce HO-1 levels upon stimulation with curcumin is a sign that aged and senescent cells are also impaired in their pathways of cellular stress responses, specially with respect to HO-1 induction, which is in accordance with reports of studies in rodents where aging affected its stimulation by different stimuli [37-40].

The antioxidant, phase II and protective enzymes that are induced by curcumin treatment have been shown to be under the influence of the cis-acting element ARE, which are regulated by the redox-sensitive transcription factor Nrf2 [24, 25]. Several studies both *in vivo* and *in vitro* have shown that, in fact, curcumin activates Nrf2 signalling [16, 20-23]. In human skin fibroblasts, we also observed an accumulation of Nrf2 in the nuclear fraction, suggesting that the induction of HO-1 and GST by curcumin was also through increased Nrf2 signaling. The increase of GSH levels and GSH/GSSG ratio observed after 24 h incubation was also attained probably through curcumin-induced increased Nrf2/ARE signaling, since the rate-limiting enzyme in

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glutathione synthesis glutamate cysteine ligase (formerly known as γ-glutamylcysteine synthetase) is also under the influence of this redox-sensitive signaling pathway [31, 41]. It is known that Keap1 controls Nrf2 stability, which under non-stressing conditions constantly targets Nrf2 for degradation, representing an attractive target for controlling Nrf2 signaling [24]. Keap1 contains cysteine residues that are thought to regulate its association with Nrf2. Since curcumin is chemically reactive and able to act as Michael reaction acceptor, its ability to increase the cellular levels of ROS or its direct potential to oxidize cysteine groups on Keap1, and thus inhibiting it, is argued as the possible mechanisms that lead to Nrf2 stabilization [17, 25, 42, 43].

A decrease in the levels and transcriptional activity of Nrf2 with age has been reported by different authors [44-46] and may explain the decrease in the levels of several antioxidant and protective enzymes and the age-related loss of GSH synthesis [44]. Experiments in human skin fibroblasts [47], and measurements in different organs of rats and mice [48-50] showed that cellular GSH/GSSG ratio declines during the aging process, due to a decrease in GSH and/or an increase in GSSG levels. Other studies also showed a decrease of antioxidant enzymes in mice astrocytes [45] and rat livers [46]. Therefore, the ability of curcumin to induce antioxidant and protective enzymes as well as GSH levels through Nrf2 signaling, besides protecting against oxidative stressors, may also delay the aging process or prevent the appearance of age-related abnormalities or diseases. In agreement with this, feeding mice with tetrahydrocurcumin (one of the major metabolites of curcumin) significantly increased their average and maximal lifespan [51]. In addition, rats fed with curcumin also present several anti-aging effects in neural tissue with increased activity of antioxidant enzymes and decreased content of lipid peroxides and lipofuscin [52].

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Therefore, it would be interesting to further test the potential of regular hormetic exposure of normal human skin fibroblasts to curcumin in order to increase cellular stress response in old cells and to afford anti-aging properties. However, care should be taken in that approach, mainly with respect to the dose and time of exposure with curcumin, since aberrant induction of oxidative stress and Akt signaling may induce premature senescence [53, 54]. Nevertheless, increased Nrf2 signaling in *Drosophila* has shown to increase significantly their oxidative stress tolerance, as well as their lifespan [55]. Thus, if induction of Nrf2 signaling can be achieved by curcumin without significant changes on Akt signaling and ROS levels, anti-aging properties may be attainted by curcumin treatment.

Basal HO-1 expression levels in senescent fibroblasts were, however, much higher than in young cells. This is in accordance with *in vivo* data in liver and brain of rodents [37-39, 56] as well as in brain of humans [57], where HO-1 expression increases with age. That may be explained because HO-1 gene is under the influence of the activity of other transcription factors that may respond differently to aging as compared with Nrf2. Actually, it was previously reported that the DNA binding activity of the hypoxia inducible factor-1 (HIF-1) increases during aging along with the increase of the expression of HIF-1-dependent genes, including HO-1 [37]. Interestingly, inhibiting phosphatidylinositol 3-kinase (PI3K) with LY for 24 h in senescent cells decreased HO-1 expression below the basal levels found in control condition. This agrees with different studies that report that PI3K/Akt pathway is involved in HIF-1 signalling [58, 59].

Another mechanism reported to be involved in Nrf2 stabilization is the phosphorylation of serine or threonine residues of the transcription factor by different kinases, such as PI3K, stress-induced MAPK and PKC [24, 25]. Different studies

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reported that curcumin stabilizes Nrf2 and/or induced HO-1 or other protective enzymes through some of these kinases [15-17, 20, 60]. The implications of kinase(s) among those studies are, however, not consistent, probably because cancer cell lines and tissues/cells from different mammalian species were used. Therefore, the use of normal human skin fibroblasts in the present study will facilitate the association of our results with human aging and the prevention of age-related diseases. In ASF-2 cells, induction of HO-1 seems to be through PI3K/Akt pathway, since p-Akt levels are increased by curcumin and inhibiting PI3K almost totally prevented curcumin-induced HO-1 expression. Interestingly, NAC that also inhibited the increase of HO-1 levels, probably by preventing the generation of oxidative stress and redox cycling by curcumin, also decreased p-Akt expression levels when compared with related controls. It seems, therefore, there is some crosstalk between redox state and PI3K/Akt pathway, which may further help to modulate Nrf2 signaling by curcumin. In fact, it is known that ROS may impact on MAPK/ERK and PI3K/Akt pathways by facilitating activation of kinase cascades and/or by inhibiting the activity of phosphatases such as PTEN [61, 62]. The protein Kras was shown to be directly activated by S-glutathionylation of a reactive thiol group due to increased levels of oxidants and trigger downstream signaling through phosphorylation of ERK and Akt [63, 64]. That may have happened with curcumin treatment as well as the inhibition of the phosphatase PTEN (a negative regulator of Akt activity), inducing Akt activity. In fact, it was also previously shown that the capacity of curcumin to induce HO-1 was accompanied by a decrease of phosphatase activities [17].

The induction of p-Akt levels was, however, largely dependent on serum stimulation during curcumin incubation. Mitogenic signals, present in the serum, stimulate the activation of receptor tyrosine kinases that results in a downstream activation of class

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I PI3K [58, 61]. PI3K activation results in phosphorylation (activation) of Akt, a reaction that can be reversed by the PIP3 phosphatase PTEN, which than modulates its cellular targets involved in cell survival, growth, proliferation and metabolism [58]. With curcumin incubation, p-Akt levels in serum-stimulated cells were increased and persisted with time. Curcumin incubation without serum stimulation did not change significantly p-Akt levels but induced the expression of HO-1, albeit to a smaller extent. This indicates that the PI3K is important in the upregulation of HO-1 expression by curcumin, but is not totally dependent on it. Therefore, the induction of a stress response by curcumin may be elevated in cells under mitogenic signaling, and in cells with potential to proliferate, such as fibroblasts. For example, during wound healing proliferation of skin fibroblasts is induced, and cells are subject to stress elicited by inflammation triggered by toxic free heme released by hemolysis, resulting in the induction of HO-1 in the injured tissue [65]. Therefore, an increased stress response in this situation elicited by curcumin may be beneficial by increasing the expression of HO-1, important in the detoxification of heme groups and in the process of wound healing [65]. In fact, it has been shown previously that curcumin enhanced the process of wound healing in cultured cells and in rats [9, 66].

In conclusion, in this report we have shown that curcumin induces cellular stress responses in normal human skin fibroblasts through generation of ROS and a decrease in cellular redox state. This curcumin-induced oxidative stress leads to a strong induction of HO-1 and other antioxidant enzymes as well as an increase in cellular GSH levels, most probably through Nrf2 signaling. This hormetic induction of stress response elicited by the hormetin curcumin led to increased protection against a further oxidant challenge, supporting the view that mild stress-induced hormesis can be applied for the modulation of aging and for improving the cellular functionality.

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Acknowledgements

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Conflict of Interests

Authors declare no conflict of interests.

For Peer Review

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Figure Legends

Figure 1. Effects of curcumin for 3 days in ASF-2 cells' viability/proliferation as measured by the MTT assay (A) or by counting the cells' number (B). Line in graphs represents the MTT reduction activity (MTT assay) or cell number (Cell counting) in the beginning of the incubation time (t = 0 h). Values are mean ± SEM of at least 3 independent experiments. * $P \leq 0.05$; ** $P \leq 0.01$ when compared with control by the one-way ANOVA.

Figure 2. Effect of curcumin on the levels of HO-1 in ASF-2 cells, at different concentrations for 24 h (B), or with 10 µM curcumin for different incubation times (C). Blots are representative of 3 independent experiments. β-Actin was used as loading control. In (A) are shown no effect of curcumin in the expression of Hsps when incubated alone for 24 h, or not even co-induction of Hsps when curcumin is present during mild heat shock (HS) of 41ºC for 1h.

Figure 3. Curcumin increases ROS levels and DNA damage in ASF-2 cells. (A) Effect of different concentrations of curcumin for 30 min in ROS levels as measured by fluorometry using the DCF probe; *t*-BOOH 100 µM was used as a positive control. (B) Effect of different concentrations of curcumin for 1 h on DNA damage measured by the alkaline version of the comet assay. (C) Representative images of the comet assay in the control and 20 µM curcumin conditions. (A & B) Values are mean ± SEM of at least 3 independent experiments. * $P \leq 0.05$; ** $P \leq 0.01$ when compared with control by the one-way ANOVA. *** $P \leq 0.001$ when compared

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with control by the Student's *t*-test. $^{+}$ $P \leq 0.05$ when compared with each other by the Student's *t*-test.

Figure 4. Curcumin-induced HO-1 levels in ASF-2 cells is prevented by NAC. (A & B) Cells were incubated with 10 μM curcumin with or without the antioxidants NAC, trolox or α-tocopherol for 8 h, and the levels of HO-1 measured by Western Blot. Blots are representative of 2 independent experiments. *β*-Actin was used as loading control. (C) Effect of NAC on the levels of HO-1 (green fluorescence) induced by curcumin for 18 h, analysed by immunofluorescence using a confocal microscope.

Figure 5. Effects of curcumin on ASF-2 cells' redox state and signalling. (A, B & C) Cells were incubated with 10 μM curcumin for different times and GSH and GSSG levels measured. Values are mean ± SEM of at least 3 independent experiments. * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$ when compared with each other by the Student's *t*-test. NS: Not significant ($P > 0.05$) when compared with each other by the Student's *t*-test. Letter notations: Groups with the same letter notation within the curcumin-treated samples are not significantly different from each other ($P > 0.05$) when analysed by the one-way ANOVA. (D) Cells were incubated with curcumin at different concentrations for 24 h and the activity of GST measured. Values are mean ± SEM of at least 3 independent experiments. ** $P \leq 0.01$ when compared with control by the one-way ANOVA. (E) Cells in 60 mm-dishes (4.5 ml) were incubated with 10 μM curcumin for different times, nuclear extracts isolated and Nrf2 expression measured by western blot. Blot is representative of 2 independent experiments.

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Figure 6. Involvement of kinases in curcumin-induced HO-1 expression in ASF-2 cells. Cells were incubated with 10 µM curcumin in the presence of different inhibitors, and after the time specified in the figures protein was extracted for western blot. Inhibitors were added 30 min before curcumin. Blots are representative of at least 2 independent experiments. β-Actin was used as loading control. (A) Effect of different kinase inhibitors on curcumin-induced HO-1 expression: PD: PD98059 50 µM; SP: SP600125 10 µM; SB: SB203580 10 µM; LY: LY294002 50 µM, STS: staurosporine 50 nM; and RO: RO320432 1.25 µM. Inhibitors alone did not induce HO-1 expression (data not shown). (B) Effect of curcumin for 8 h on the active (phosphorylated) form of different kinases. (C) Effect of curcumin in the presence of NAC or LY in the expression of phospho (p)-Akt after 2 and 8 h of incubation. (D) Phospho-Akt expression along the time after medium change in the absence or presence of 10 µM curcumin. (E) Left panel: Effect of medium change and curcumin on the levels of phospho-Akt and HO-1; Right panel: Effect of different components of the medium on p-Akt levels 2 h after replacing the medium.

Figure 7. Hormetic effects of curcumin in ASF-2 cells. (A) Cells were incubated with 10 µM curcumin for 2 h, followed by a recovery period for different times with fresh medium, and the expression of HO-1 measured by western blot. Blots are representative of 2 independent experiments. β-Actin was used as loading control. (B) Cells were incubated with 10 µM curcumin for 2 h, followed by a recovery period of 16 h with fresh medium, and GSH and GSSG levels measured as described under Materials and Methods. Values are mean ± SEM of 3 independent experiments. ** $P \leq 0.01$ when compared with the respective control by the Student's *t*-test. (C) Cells were incubated with 10 or 20 µM curcumin for 2 h, followed by a recovery period of

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16 h with fresh medium. Then, cells were incubated with 200 μM *t*-BOOH for 3 h and cell viability measured by the LDH leakage method. Values are mean ± SEM of at least 4 independent experiments. *** $P \leq 0.001$ when compared with control by the Student's *t*-test. $^{+}$ $P \leq 0.05$, $^{++}$ $P \leq 0.01$ when compared with *t*-BOOH alone by the one-way ANOVA.

Figure 8. Effects of curcumin on the levels of HO-1 in senescent ASF-2 cells. (A) Effect of curcumin on HO-1 levels in young and senescent cells. Senescent cells were incubated with curcumin at different concentrations for 24 h (B), or with 10 μM curcumin for different incubation times (C), and the expression of HO-1 and p-Akt measured by western blot. (D) Effects of curcumin in the presence of NAC or LY on the levels of p-Akt and HO-1 after 2, 8 and 24 h of incubation. Blots are representative of 2 independent experiments. β-Actin was used as loading control.

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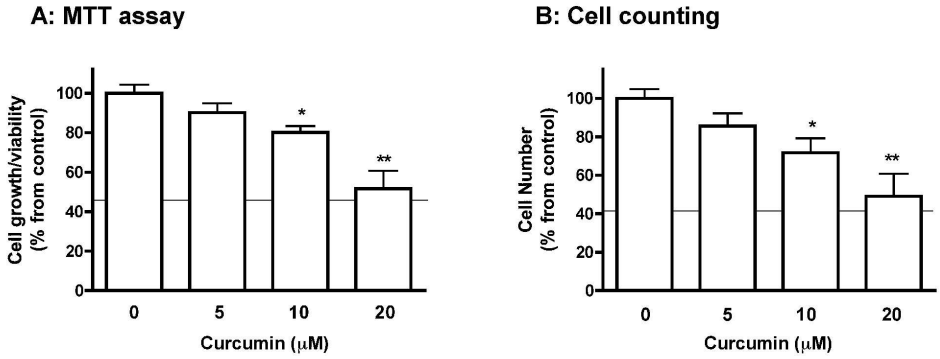

Figure 1. Effects of curcumin for 3 days in ASF-2 cells' viability/proliferation as measured by the MTT assay (A) or by counting the cells' number (B). Line in graphs represents the MTT reduction activity (MTT assay) or cell number (Cell counting) in the beginning of the incubation time (t = 0 h). Values are mean ± SEM of at least 3 independent experiments. * P≤0.05; ** P≤0.01 when compared with control by the one-way ANOVA.
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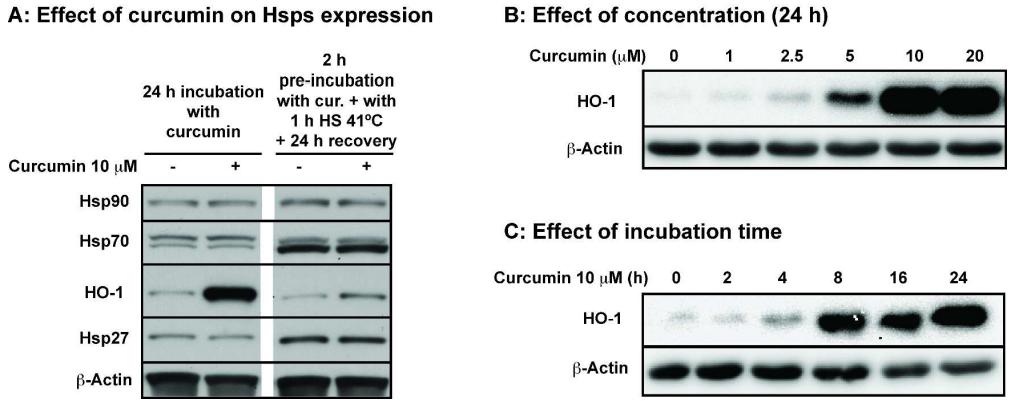

Figure 2. Effect of curcumin on the levels of HO-1 in ASF-2 cells, at different concentrations for 24 h (B), or with 10 μM curcumin for different incubation times (C). Blots are representative of 3 independent experiments. β-Actin was used as loading control. In (A) are shown no effect of curcumin in the expression of Hsps when incubated alone for 24 h, or not even co-induction of Hsps when curcumin is present during mild heat shock (HS) of 41ºC for 1h.
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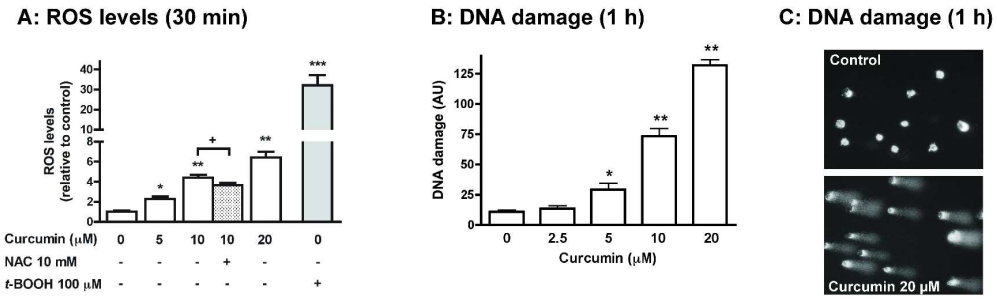

Figure 3. Curcumin increases ROS levels and DNA damage in ASF-2 cells.
(A) Effect of different concentrations of curcumin for 30 min in ROS levels as measured by fluorometry using the DCF probe; t-BOOH 100 μM was used as a positive control. (B) Effect of different concentrations of curcumin for 1 h on DNA damage measured by the alkaline version of the comet assay. (C) Representative images of the comet assay in the control and 20 μM curcumin conditions. (A & B) Values are mean ± SEM of at least 3 independent experiments. * P≤0.05; ** P≤0.01 when compared with control by the one-way ANOVA. *** P≤0.001 when compared with control by the Student's t-test. + P≤0.05 when compared with each other by the Student's t-test.
147x43mm (600 x 600 DPI)

eer Review

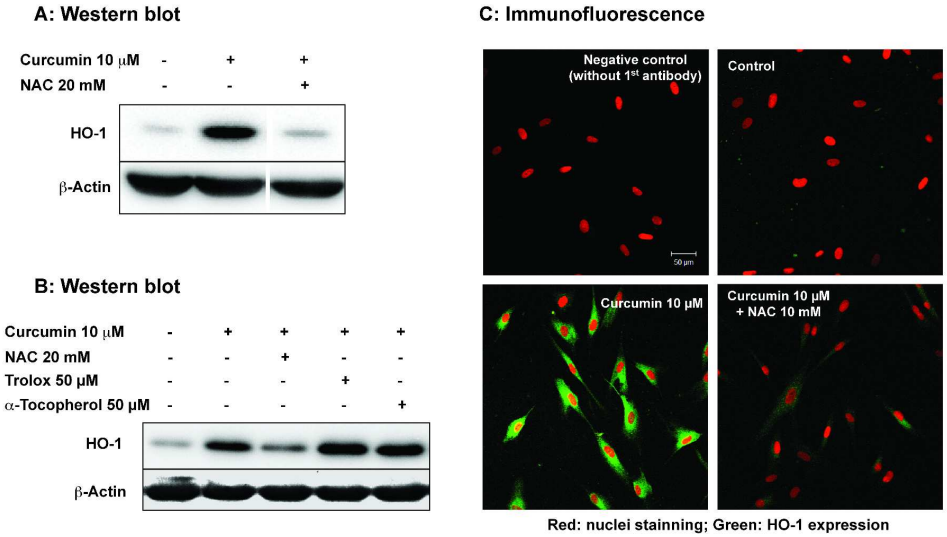

Figure 4. Curcumin-induced HO-1 levels in ASF-2 cells is prevented by NAC.
(A & B) Cells were incubated with 10 μM curcumin with or without the antioxidants NAC, trolox or α-tocopherol for 8 h, and the levels of HO-1 measured by Western Blot. Blots are representative of 2 independent experiments. β-Actin was used as loading control. (C) Effect of NAC on the levels of HO-1 (green fluorescence) induced by curcumin for 18 h, analysed by immunofluorescence using a confocal microscope.
320x180mm (300 x 300 DPI)

For Review

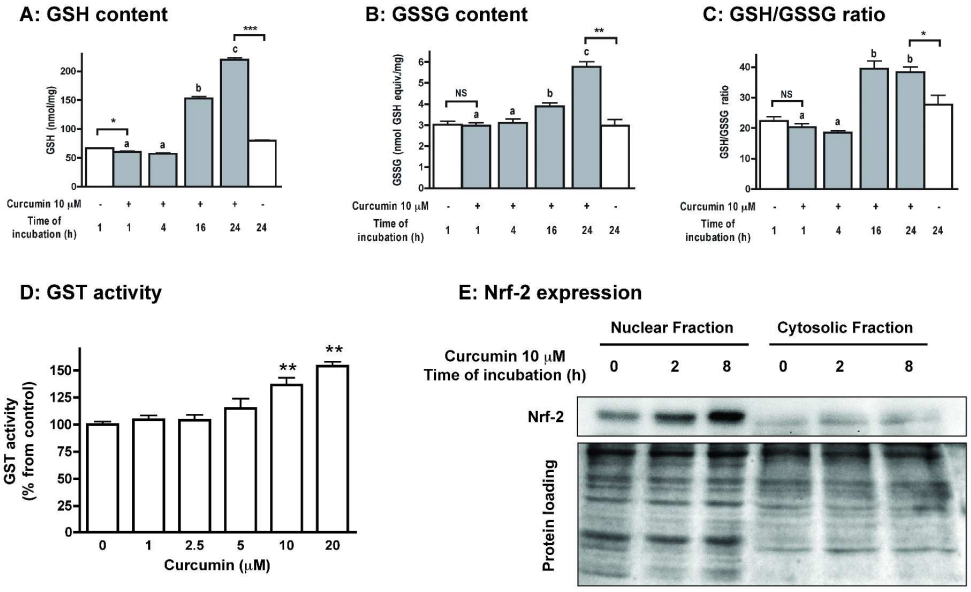

Figure 5. Effects of curcumin on ASF-2 cells' redox state and signalling.
(A, B & C) Cells were incubated with 10 μM curcumin for different times and GSH and GSSG levels measured. Values are mean ± SEM of at least 3 independent experiments. * P≤0.05; ** P≤0.01; *** P≤0.001 when compared with each other by the Student's t-test. NS: Not significant (P>0.05) when compared with each other by the Student's t-test. Letter notations: Groups with the same letter notation within the curcumin-treated samples are not significantly different from each other (P>0.05) when analysed by the one-way ANOVA. (D) Cells were incubated with curcumin at different concentrations for 24 h and the activity of GST measured. Values are mean ± SEM of at least 3 independent experiments. ** P≤0.01 when compared with control by the one-way ANOVA. (E) Cells in 60 mm-dishes (4.5 ml) were incubated with 10 μM curcumin for different times, nuclear extracts isolated and Nrf2 expression measured by western blot. Blot is representative of 2 independent experiments.
316x185mm (300 x 300 DPI)

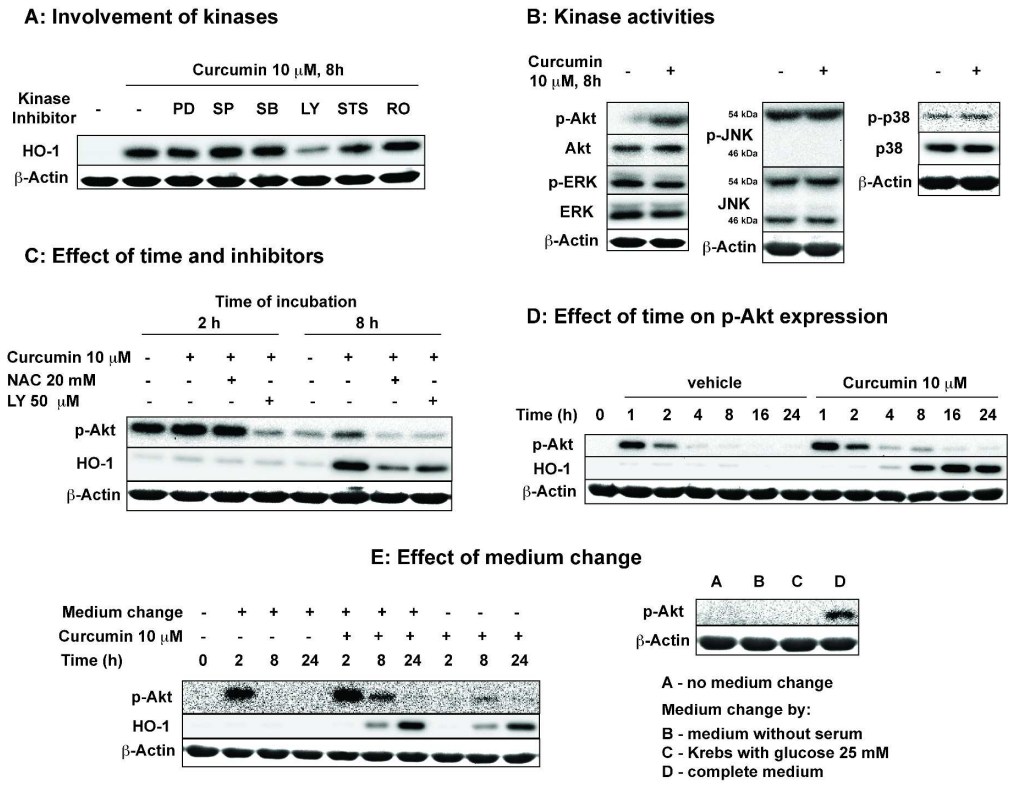

Figure 6. Involvement of kinases in curcumin-induced HO-1 expression in ASF-2 cells. Cells were incubated with 10 μM curcumin in the presence of different inhibitors, and after the time specified in the figures protein was extracted for western blot. Inhibitors were added 30 min before curcumin. Blots are representative of at least 2 independent experiments. β-Actin was used as loading control. (A) Effect of different kinase inhibitors on curcumin-induced HO-1 expression: PD: PD98059 50 μM; SP: SP600125 10 μM; SB: SB203580 10 μM; LY: LY294002 50 μM, STS: staurosporine 50 nM; and RO: RO320432 1.25 μM. Inhibitors alone did not induce HO-1 expression (data not shown). (B) Effect of curcumin for 8 h on the active (phosphorylated) form of different kinases. (C) Effect of curcumin in the presence of NAC or LY in the expression of phospho (p)-Akt after 2 and 8 h of incubation. (D) Phospho-Akt expression along the time after medium change in the absence or presence of 10 μM curcumin. (E) Left panel: Effect of medium change and curcumin on the levels of phospho-Akt and HO-1; Right panel: Effect of different components of the medium on p-Akt levels 2 h after replacing the medium.
308x239mm (300 x 300 DPI)

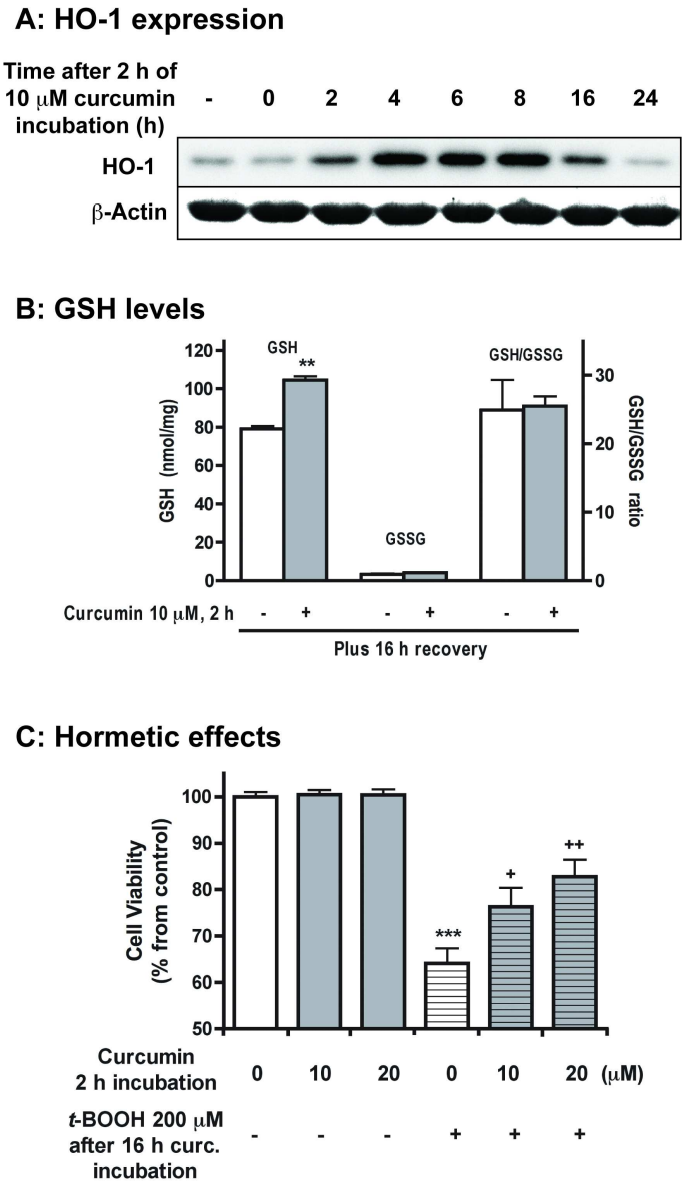

Figure 7. Hormetic effects of curcumin in ASF-2 cells. (A) Cells were incubated with 10 μM curcumin for 2 h, followed by a recovery period for different times with fresh medium, and the expression of HO-1 measured by western blot. Blots are representative of 2 independent experiments. β-Actin was used as loading control. (B) Cells were incubated with 10 μM curcumin for 2 h, followed by a recovery period of 16 h with fresh medium, and GSH and GSSG levels measured as described under Materials and Methods. Values are mean ± SEM of 3 independent experiments. ** P≤0.01 when compared with the respective control by the Student's t-test. (C) Cells were incubated with 10 or 20 μM curcumin for 2 h, followed by a recovery period of 16 h with fresh medium. Then, cells were incubated with 200 μM t-BOOH for 3 h and cell viability measured by the LDH leakage method. Values are mean ± SEM of at least 4 independent experiments. *** P≤0.001 when compared with control by the Student's t-test. + P≤0.05, ++ P≤0.01 when compared with t-BOOH alone by the one-way ANOVA.

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For Peer Review

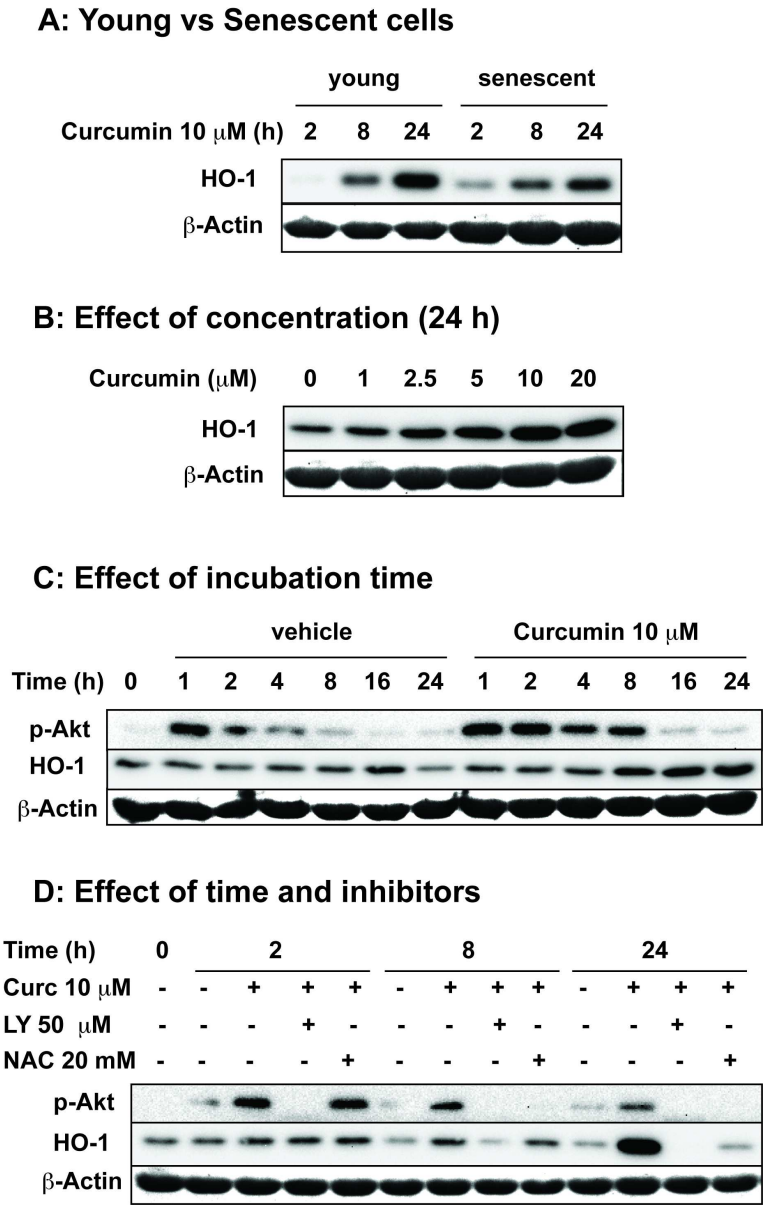

Figure 8. Effects of curcumin on the levels of HO-1 in senescent ASF-2 cells.
(A) Effect of curcumin on HO-1 levels in young and senescent cells. Senescent cells were incubated with curcumin at different concentrations for 24 h (B), or with 10 μM curcumin for different incubation times (C), and the expression of HO-1 and p-Akt measured by western blot. (D) Effects of curcumin in the presence of NAC or LY on the levels of p-Akt and HO-1 after 2, 8 and 24 h of incubation. Blots are representative of 2 independent experiments. β-Actin was used as loading control.
156x247mm (300 x 300 DPI)