

# Curcumin's Biphasic Hormetic Response on Proteasome Activity and Heat-Shock Protein Synthesis in Human Keratinocytes

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**ABSTRACT:** Curcumin (diferuloylmethane), is a component of the yellow powder prepared from the roots of *Curcuma longa* (Zingiberaceae), also known as tumeric or turmeric. It is widely cultivated and used as a food ingredient in tropical areas of Asia and Central America. Treatment of mid-passage human epidermal keratinocytes with curcumin resulted in a biphasic hormetic dose-response with respect to proteasome activity. Curcumin treatment (up to 1  $\mu\text{M}$  for 24 h) increased chymotrypsin-like activity by 46% compared to that in untreated keratinocytes. However, higher concentrations of curcumin were inhibitory, and at 10  $\mu\text{M}$  the proteasome activity decreased to 46% of its initial value. Furthermore, the preincubation of human keratinocytes at 43°C for 1 h, followed by 24-h treatment with 3  $\mu\text{M}$  curcumin, led to an increase in heat-shock protein (hsp70 and hsp90) levels by 24% and 19%, respectively, and the effect was sustained at concentrations up to 10  $\mu\text{M}$ . On the other hand, the level of the small hsp27 was unaffected by curcumin concentrations of 0.3–1  $\mu\text{M}$ , while it decreased by 34% at 10  $\mu\text{M}$ .

**KEYWORDS:** aging; hormesis; hormetin; heat shock; stress; protein degradation

The use of medicinal plants in pharmacology has increased significantly in recent times, owing to their affordability and apparent safety, when compared to synthetic drugs. Curcumin (diferuloylmethane), the active constituent of *Curcuma longa*, is one of the best studied natural antioxidant compounds. Curcumin has been used as a spice and coloring agent for centuries, and it is used in India against hepatic disorders, anorexia, diabetic wounds, and rheumatism.<sup>1</sup> Curcumin has been shown to have anti-inflammatory, anti-carcinogenic,

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Ann. N.Y. Acad. Sci. 1067: 394–399 (2006). © 2006 New York Academy of Sciences.  
doi: 10.1196/annals.1354.056

anti-diabetogenic, antibacterial, antiviral, and antioxidant effects.<sup>2,3</sup> Curcumin has also been shown to induce the heat-shock (HS) response in HeLa cells in a time-dependent and dose-dependent manner,<sup>4</sup> and an enhancement of HS response has been seen in rat liver cells and Swiss 3T3 mouse fibroblasts.<sup>5</sup> In addition, high concentrations of curcumin were reported to inhibit proteasome activity in HeLa cells.<sup>6</sup> Proteasome inhibition was also found to induce HS response of mammalian cells,<sup>7</sup> suggesting that the two mechanisms are related. Therefore, the present investigation was undertaken to test whether curcumin modulates HS response and proteasome activity in cultured human keratinocytes undergoing aging *in vitro*.

## EXPERIMENTAL METHODS

Primary cultures of normal diploid human epidermal keratinocytes were established from mammary skin biopsies obtained from a healthy woman donor (age: 28 years). Cells were grown in T<sub>25</sub> plastic flasks (COSTAR, Cambridge, UK) at 37°C, 5% CO<sub>2</sub> and 95% humidity in Epilife medium (Cascade Biologics, Mansfield, UK) supplemented with 100 ng/mL EGF, 0.18 mg/mL hydrocortisone, 2.4 mg/mL insulin, 2.5 mg/mL transferrin, 0.06 M CaCl<sub>2</sub>, and 12 mg/mL BPA. The medium was changed twice a week, and when the cells reached 80% confluence, the culture was split using the trypsin/EDTA (Biowhittacker™ Cambex Bioscience, Verviers, Belgium) method.

Curcumin was purchased from Sigma-Aldrich (St Louis, MO, USA; catalog number: C1386). The naturally occurring ratio for curcuminoids is 5% bisdesmethoxycurcumin, 15% desmethoxycurcumin, and 80% curcumin.<sup>8</sup> A stock solution of curcumin (10 mM; molecular weight 368.39) was dissolved in 100% DMSO. During incubation of cells with curcumin, the final DMSO concentration was 0.08%. Cell survival after exposure to different curcumin concentrations was measured with the 3-(4,5 dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay.

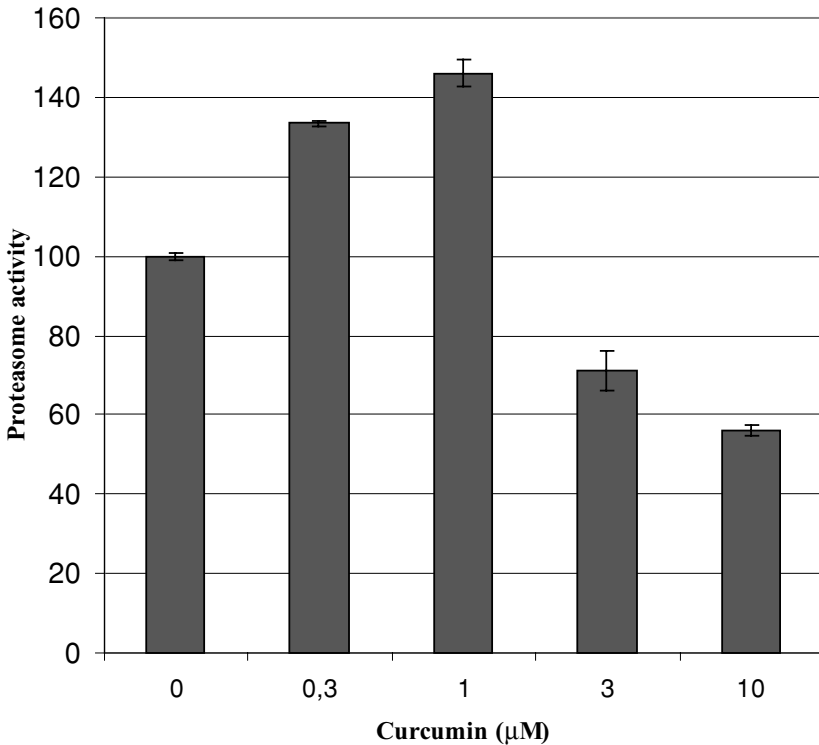
For proteasome activity assay, cells were washed with cold PBS and harvested using cell scraper and buffer A (50 mM NaCl, 10 mM HEPES, pH 8, 250 mM sucrose, 1 mM EDTA, and 0.2% Triton X-100). Cell suspension was vortexed for 2 min and centrifuged at 15,000 rpm at 4°C. Twenty micrograms of total protein were added to a 96-well plate and mixed with 25 μM Suc-LLVY-AMC (Sigma-Aldrich) in 200 μL 0.1 M HEPES pH 7.4. As a negative control, 800 μM MG132 (Sigma-Aldrich) was incubated with the sample and substrate. The fluorescence intensity was measured at excitation 360 nm and emission 460 nm for 30 min at 37°C, using a fluorimeter (BMG LABTECH, GmbH, Hoffenberg, Germany). The proteolytic activities were expressed as a percentage of control.

For heat-shock response, cells were subjected to 43°C HS for 1 h, treated with curcumin for 24 h, then harvested in 500 μL lysis buffer (5M NaCl, 1M MgCl<sub>2</sub>,

50% glycerol, 0.5% SDS, 1 M Tris/HCl, pH 8). Cellular proteins were isolated and protein content of the cell extracts was determined by the Lowry method. All samples were heated for 3 min at 95°C. The proteins were transferred to polyvinylidene fluoride (PVDF) membrane (Immobilon-P, Millipore). The membranes were blocked overnight at 4°C in TBS-T containing 5% skim milk, and then incubated with primary antibodies, hsp90, hsp70, and hsp27 (Nordic Biosite, Sweden) for 60 min at room temperature.

## RESULTS

Serially passaged middle-aged human epidermal keratinocytes (~50% life-span completed) were treated with curcumin for 24 h. Curcumin concentrations up to 1  $\mu$ M did not affect cell viability, whereas at high concentrations (3–10  $\mu$ M) cell viability decreased to 48% and 53%, respectively (data not shown). FIGURE 1 depicts the effect of curcumin on the chymotrypsin-like



**FIGURE 1.** Effect of curcumin on chymotrypsin-like proteasomal activity of mid-passage human keratinocytes (50% life span completed). Data are mean  $\pm$  SD of triplicate measurements and expressed as a percentage of control, measured in the absence of curcumin.

activity of middle-aged cultured keratinocytes, showing that at 0.3  $\mu\text{M}$  and 1  $\mu\text{M}$  curcumin stimulated proteasome activity by 34% and 46%, respectively. On the other hand, significant inhibition of proteasome activity (32% and 46%) was observed at 3 and 10  $\mu\text{M}$  curcumin concentration. To investigate the effect of curcumin on HS response, cells were preincubated at 43°C for 1 hr followed by 24-h curcumin treatment. In HS-exposed cells, treatment with 3  $\mu\text{M}$  curcumin resulted in a marked increase in the levels of hsp90 and hsp70 (19% and 24%, respectively) as compared with HSP levels in untreated cells and this effect of curcumin was additive (data not shown). However, hsp27 levels were not affected by curcumin treatment at concentrations up to 1  $\mu\text{M}$ , and were reduced by 34% at 10  $\mu\text{M}$ .

## DISCUSSION

Inhibition of the proteasome by application of high (up to 50  $\mu\text{M}$ ) curcumin concentrations was previously reported<sup>6</sup> but the effect of lower concentrations has not been investigated. Our studies have shown that low (up to 1  $\mu\text{M}$ ) concentrations of curcumin have a stimulatory effect on proteasome in human keratinocytes, whereas high concentrations of curcumin are inhibitory for the proteasome. We have also observed similar biphasic response of curcumin on the proteasomal activity in other cell types, including human fibroblasts and telomerase-immortalized mesenchymal bone marrow stem cells (data not shown). This biphasic dose response is a typical expression of hormesis, in which low doses of a compound elicit beneficial biological effects, whereas higher doses do have deleterious effects.<sup>9-11</sup> This phenomenon has been shown in other conditions such as irradiation, temperature, hypergravity, pro-oxidants, electric shocks and repeated physical injuries, which have been reported to have several beneficial effects including an extension of life span.<sup>9-11</sup>

With respect to curcumin's effects as a modulator of HS response, previous studies on C6 rat glioma cells had shown that curcumin doses (up to 30  $\mu\text{M}$ ) increased the levels of hsp27, hsp70, and  $\alpha\text{B}$  crystallin.<sup>5</sup> Furthermore, it was shown that proteasome inhibition induced hsp70 and hsp27 expression in mouse embryonic fibroblasts (MEF) cells immortalized by SV-40 transfection.<sup>12</sup> We have also observed in mid-passage human keratinocytes that the synthesis of hsp70 and hsp90, but not of hsp27, was enhanced after preincubation at 43°C for 1 h followed by 24-h curcumin treatment. In this study, the 32% decrease in proteasome activity induced by 3  $\mu\text{M}$  curcumin was accompanied with an increase in the levels of hsp70 and hsp90 by 24% and 19%, respectively. As previously discussed, it seems that the induction of hsp locks 20S proteasome in a latent inactive state and impairs further activation of the 26S proteasome by ATP.<sup>13</sup>

In conclusion, our studies have shown that curcumin modulates the HS response and proteasome activity in cultured human keratinocytes in a biphasic

hormetic manner. Thus, curcumin may be a useful “hormetin” as a natural food component by enhancing protective stress response and by stimulating proteasome-mediated removal of abnormal proteins during aging. Further studies are in progress to determine the mechanism of curcumin’s hormetic effects on human cells undergoing aging *in vivo*.

### ACKNOWLEDGMENTS

David C. Kraft is acknowledged for his help in the proteasome assay. Ulrich Berge and Regina Gonzalez-Dosal are acknowledged for useful discussions. Thanks are extended to Anna Gylling, Helle Jackobsen, and Gunhild Siboska for expert technical assistance. The Laboratory of Cellular Ageing is supported by research grants from The Danish Research Councils, Carlsberg Fund, Senetek PLC, and EU’s Biomed Health Programmes.

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