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Anti-Inflammatory and Pro-apoptotic Effects of Curcumin and Resveratrol on the Human Lung Fibroblast Cell Line MRC-5

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Abstract

Background: The naturally occuring polyphenols curcumin and resveratrol are considered to be powerful antioxidants and anti-inflammatory compounds and both inhibit the proliferation of different types of cancer cells. In the present study, we investigated possible anti-inflammatory and pro-apoptotic effects of curcumin and resveratrol on the human lung fibroblast cell line MRC-5.

Methods: MRC-5 cells were stimulated for 6 h with interleukin (IL)-1β or phorbol 12- myristate 13-acetate (PMA) in the absence or presence of different concentrations of curcumin or resveratrol. The release of interleukin (IL)-6 was quantified by enzyme-linked immunosorbent assay (ELISA). The modulation in phosphorylation of the transcription factor nuclear factor-kappa B (NF-κB) and mitogen-activated protein kinases (MAPKs) such as p^{38} and ERK1/2 were analyzed by Western blot. Cytotoxic and pro-apoptotic effects of curcumin and resveratrol were monitored by the measurement of lactate dehydrogenase (LDH) activity and by Annexin-V/7-AAD staining.

Results: Both curcumin and resveratrol effectively attenuated IL-1β and PMA-induced IL-6 expression in MRC-5 cells. Furthermore, curcumin treatment induced apoptosis via caspase-3 signaling and caused endoplasmic reticulum (ER) stress. Salubrinal, an inhibitor of serine/threonine phosphatase PP1, and antioxidants such as N-acetyl-cysteine (NAC), reduced glutathione (GSH) and sodium hydrogen sulfide (NaHS) diminished the cytotoxic effects of curcumin on MRC-5 cells. In contrast to curcumin, resveratrol had no negative effects on cell viability.

Keywords: MRC-5 cells; Curcumin; Resveratrol; IL-6; Apoptosis.

Introduction

Curcumin (diferuloylmethane) is a yellow pigment found in the rhizome of turmeric (*Curcuma longa L., Zingiberaceae*) with a wide range of pharmacological activities [1,2]. Antioxidant, anti-carcinogenic, anti-inflammatory and pro-apoptotic effects of this compound have been assessed in various *in vitro* and *in vivo* systems [3]. Numerous studies have demonstrated its role in suppressing expression of the cell cycle protein cyclin D1 and transcription factors that are implicated in carcinogens is such as nuclear factor kappa-B (NF-κB) and activator protein-1 (AP-1). Furthermore, curcumin induced programmed cell death (apoptosis) in tumor cells by activating caspases and down-regulating Bcl-2 family proteins [4,5]. Recent reports have also demonstrated that curcumin inhibits proteosomal activity [6], triggers accumulation of cytosolic Ca^{2+} [7], and disrupts protein disulfide bond formation [8], all of which initiate ER stress response.

Resveratrol (*trans*-3,4,9,5-trihydroxystilbene) is a natural phytoalexin found in large quantities in grapes and other food products [9,10]. Resveratrol was found to have a potent anti-carcinogenic activity in several animal models of cancer [9]. The anti-carcinogenic properties of resveratrol are closely associated with its antioxidant activity and the ability to inhibit cyclooxygenase-2 (COX-2), hydroperoxidase, protein kinase C (PKC), Bcl-2 phosphorylation, Akt, NF-κB, matrix metalloprotease-9 (MMP-9), and programmed cell death [11-14].

In the present study, we investigated possible anti-inflammatory and pro-apoptotic properties of curcumin and resveratrol in the human lung fibroblast cell line MRC-5. Data demonstrate that both curcumin and resveratrol effectively blocked interleukin (IL)-1β and phorbol 12-myristate 13-acetate (PMA)-induced interleukin (IL)-6 expression in MRC-5 cells. Furthermore, curcumin suppressed activation of NF-κB and inhibited degradation of IκBα, respectively. Beside its potent anti-inflammatory properties, curcumin, at high concentrations, had pronounced cytotoxic effects on MRC-5 cells, induced apoptosis and triggered ER stress response via activation of caspase-3 and phosphorylation of translation initiation factor 2 (eIF-2α). In strong contrast to curcumin, resveratrol did not negatively influence cell viability.

Materials and Methods

Reagents

Unless stated otherwise, all reagents were from Sigma Aldrich (Vienna, Austria) and C Roth (Karlsruhe, Germany). IL-1β was purchased from ProSpecTany Technogene (Tel Aviv, Israel). Antibodies for p38 MAPK and ERK1/2, NF-κB, IκBα, caspase-3/-7/-9, and eIF-2α were from Cell Signaling (New England Biolabs, Frankfurt am Main, Germany).

Cell culture and experimental methods

The human lung fibroblast cell line MRC-5 was kindly provided by B Lohberger (Department of Orthopaedics, Medical University of Graz, Austria). Cells were cultivated in Dulbecco′s modified Eagle′s medium (DMEM) supplemented with 10% heat-inactivated foetal bovine serum (FBS), 50 IU/ml penicillin, 50 µg/ml streptomycin, and 2 mmol/l-L-glutamine in a humidified incubator with 5% CO_2 and 95% air. Stock solutions of curcumin and resveratrol were prepared with ethanol (96% v/v) and diluted properly into medium. To induce IL-6 expression, MRC-5 cells were stimulated for 6 h either with IL-1β (10 ng/ml) or PMA (100 ng/ml). All experiments were performed in duplicates and were repeated twice.

Lactatdehydrogenase (LDH) assay

MRC-5 cells were seeded in 12-well cell culture plates (2 x 105/well) and cultured overnight in DMEM plus 10% (v/v) FBS. The next day, culture medium was changed and replaced by DMEM containing 1% FBS (v/v) lacking phenol red. Curcumin and resveratrol were added and cells were incubated for another 6 h. LDH activity in cell culture supernatants was determined with a commercially available kit (Roche, Vienna, Austria).

Annexin-V/7-AAD-assay

MRC-5 cells were cultured overnight in 6-well cell culture plates (6 x 105/well) in DMEM supplemented with 10% (v/v) FBS. The next day, culture medium was changed and curcumin or resveratrol were added to the desired concentrations and cells were incubated for another 6 h. Then, cells were trypsinized and labeled with the Muse™ Annexin-V & Dead Cell reagent (Merck-Millipore, Germany). Detection of living cells, cells in the early or late apoptotic stage and dead cells was performed with the Muse™ Cell Analyzer (Merck-Millipore, Germany).

Cytokine measurement

Ready-to-go enzyme-linked immunosorbent assay (ELISA) kit from ebioscience (Vienna, Austria) was used to quantify IL-6 levels in cell culture supernatants.

Gel electrophoresis and Western blot analysis

SDS-PAGE and Western blots were performed by standard methods. Briefly, at the end of the experiment, cells were washed twice with PBS (p^H 7.4) and lysed with SDS-loading buffer. Cell lysates were boiled for 5 min at 95°C and treated with a short pulse of ultrasound (10 sec) to minimize viscosity. Equal amounts were then loaded onto SDS gels. Proteins were separated on 12% polyacrylamide mini gels, blotted by semi-dry electrotransfer to nitrocellulose membranes and probed with specific antibodies. Proteins were visualized using Roti-Lumin (Roth, Karlsruhe, Germany) and the GeneGnome chemiluminescence detection device (Syngene, Cambridge, UK).

Statistical analysis

Statistical analysis was performed using GraphPad Prism version 5.04. Data were expressed as means ± SEM. Differences between three or more groups were calculated by 1 and 2-way ANOVA, respectively. A probability level of 0.05 was used to define significance.

Results

Curcumin and resveratrol attenuate IL-1β and PMA-induced IL-6 expression

To evaluate possible anti-inflammatory properties of curcumin and resveratrol, MRC-5 cells were stimulated for 6 h with IL-1β or PMA in the absence or presence of increasing concentrations of curcumin or resveratrol (12.5–100 µM). As shown in Figure 1A, IL-1β induced IL-6 expression in MRC-5 cells to a high extent (~8 ng/ml). IL-6 production induced by PMA was much less pronounced, approximately 20-fold lower (Figure 1B). Data show that relatively low concentrations of curcumin (12.5 µM) almost completely blocked IL-6 expression, regardless of whether IL-1β or PMA was used for stimulation (Figure 1A and 1B). The inhibitory effect of curcumin on IL-6 expression was much higher compared to that of the same concentration of resveratrol (95 versus 15% inhibition at 12.5 µM; Figure 1A). In fact, 100 µM resveratrol was required to obtain the same degree of suppression of IL-6 production as observed with 12.5 µM curcumin. The half maximal inhibitory concentration (IC_{50}) of curcumin on IL-6 expression was estimated by a dose-response curve; IC_{50} ~ 5 µM (Figure 1C). Next, we investigated whether curcumin may amplify the inhibitory effects of resveratrol on IL-6 expression. MRC-5 cells were stimulated for 6 h with IL-1β in the absence or presence of resveratrol or resveratrol plus curcumin. As shown in Figure 1D, 50 µM resveratrol reduced IL-6 expression at about 60%, whereas resveratrol in combination with 5 µM curcumin blocked IL-6 release almost completely (~95%) indicating that resveratrol and curcumin have additive effects.

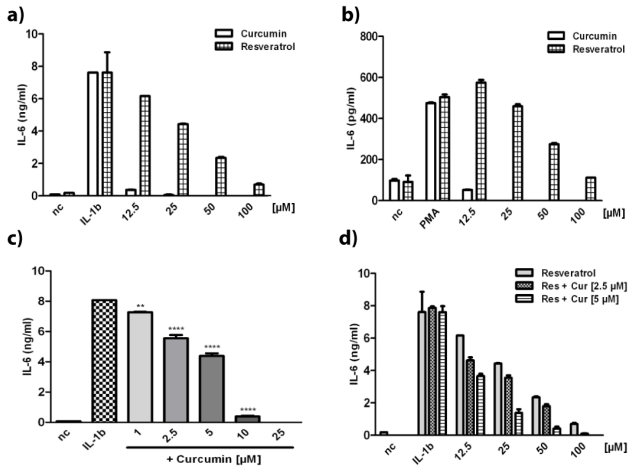

Figure 1: Curcumin and resveratrol diminish IL-1β and PMA-induced IL-6 expression in MRC-5 cells. Cells were stimulated for 6 h with IL-1β or PMA in the absence or presence of indicated concentrations of curcumin or resveratrol (A, B). (C) Dose-response curve of curcumin. MRC-5 cells were stimulated for 6 h with IL-1β in the absence or presence of indicated concentrations of curcumin. (D) MRC-5 cells were stimulated for 6 h with IL-1β in the absence or presence of resveratrol or resveratrol plus curcumin. IL-6 levels in cell culture supernatants were quantified by ELISA. Data from 2 independent experiments are presented as mean ± SEM (**p<0.01; ****p<0.0001).

Curcumin blocks activation of NF-κB

Next, we tried to identify potential signaling pathways (NF-κB, etc.) affected by curcumin and resveratrol. MRC-5 cells were pre-incubated for 30 min with curcumin or resveratrol (12.5–100 μM) before being stimulated for 20 min with IL-1β. Activation of NF-κB (phosphorylation of the p^{65} subunit at serine 536) and degradation of IκBα, respectively, were analyzed by Western blot. As shown in Figure 2A, NF-κB was rapidly phosphorylated after the addition of IL-1β. Interestingly, phosphorylation of NF-κB and degradation of IκBα were blocked only at 100 μM curcumin, lower concentrations (12.5–50 μM), however, were inefficient (Figure 2A). In comparison to curcumin, resveratrol completely failed to inhibit NF-κB activation (Figure 2B).

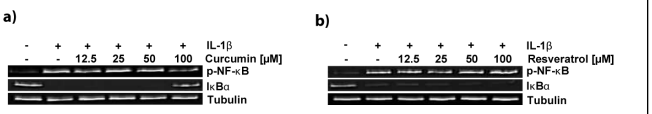

Figure 2: Curcumin inhibits phosphorylation of NF-κBp^{65} in IL-1β-stimulated MRC-5 cells. Cells were pre-incubated for 30 min with indicated concentrations of curcumin or resveratrol before being stimulated for 20 min with IL-1β. Total cell proteins were subjected to SDS-PAGE electrophoresis and blotted onto nitrocellulose membranes. Phospho-NF-κB (p-NF-κB) and IκBα were detected with specific antibodies; (A) Cells were stimulated with IL-1β -/+ curcumin; (B) Cells were stimulated with IL-1β -/+ resveratrol.

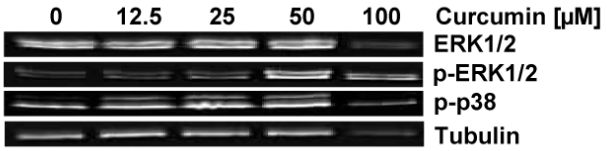

Figure 3: Curcumin induces phosphorylation of ERK1/2. MRC-5 cells were treated for 60 min with indicated concentrations of curcumin or resveratrol. Total cell proteins were subjected to SDS-PAGE electrophoresis and blotted onto nitrocellulose membranes. Phospho-ERK1/2 (p-ERK1/2) and phospho-p^{38} MAPK (p-p^{38}) were detected with specific antibodies against Thr202/Tyr204 (p-ERK1/2) and Thr180/Tyr182 (p-p^{38}); (A), Curcumin-treated cells; (B), Resveratrol-treated cells.

Curcumin activates extracellular signal-regulated kinase (ERK)-1/2

Besides NF-κB, mitogen-activated protein kinases (MAPKs) play a central role in proliferation, gene expression, differentiation, cell survival, and apoptosis. Therefore, we investigated the effects of curcumin and resveratrol on MAPK activation (p^{38} MAPK and ERK1/2) in MRC-5 cells. Cells were treated for 60 min with different concentrations of curcumin or resveratrol (12.5–100 μM) and phosphorylation of p^{38} MAPK and ERK1/2 was analyzed by Western blot. As shown in Figure 3, phosphorylation of ERK1/2 was induced at 50 μM curcumin, whereas lower concentrations did not affect ERK1/2. Notably, treatment of MRC-5 cells with 100 μM curcumin led to a dramatic decrease of total protein content (Figure 3) indicating that curcumin at high concentrations had cytotoxic effects. In contrast to ERK1/2, p^{38} MAPK was only slightly affected (Figure 3).

Curcumin induces cell death through apoptosis

To analyze cytotoxic and pro-apoptotic effects of curcumin and resveratrol, we measured lactate dehydrogenase (LDH) activity in cell culture supernatants and performed Annexin-V/7-AAD staining of curcumin and resveratrol-treated cells. Treatment of MRC-5 cells with 25 μM curcumin led to a significant increase in LDH activity (Figure 4A) whereas resveratrol, however, did not. Next, we asked whether cell death observed in curcumin-treated cells was due to apoptosis. MRC-5 cells were incubated for 6 h with increasing concentrations of curcumin (12.5–100 μM) and apoptotic activity was analyzed by Annexin-V/7-AAD staining. Data show that treatment with 12.5 μM curcumin had no significant effect on cell viability (Figure 4B). At 25 μM curcumin, about 40% of the total cell population was in the early apoptotic stage, at 50 μM, already 80%. At 100 μM curcumin, 90% of the cells were in the late apoptotic/dead stage (Figure 4B). In contrast, treatment of MRC-5 cells with resveratrol (up to 24 h) did not negatively affect cell viability (data not shown). In addition, we investigated whether apoptosis was induced via the caspase signaling pathway. As shown in Figure 4C, degradation of pro-caspase-3 was evident 1 h post-treatment. Interestingly, a decrease of pro-caspase-7/-9 could not be detected (Figure 4C). Confirming the data shown in Figure 3, a dramatic decline in total protein content was observed at 100 μM curcumin (Figure 4C) suggesting that curcumin at high concentrations led to irreversible cell damage. In cells treated with resveratrol, however, caspases were not activated (Figure 4E).

Curcumin induces endoplasmic reticulum (ER) stress

Phosphorylation of the alpha subunit of the eukaryotic initiation factor-2 (eIF-2α) is a well-documented mechanism of down-regulating protein synthesis triggered by agents that induce apoptosis, ER stress and protein misfolding. To clarify whether curcumin may also induce ER stress in MRC-5 cells, cells were left untreated or were exposed for 30 min to curcumin (6.25–100 μM). As shown in Figure 5A, treatment of cells with 100 μM curcumin resulted in marked phosphorylation of eIF-2α. Salubrinal (Sal) as an inhibitor of serine/threonine phosphatase PP1 blocks eIF-2α dephosphorylation. When MRC-5 cells were treated for 6 h with 25 μM curcumin in the presence of Sal (50 μM), the survival rate significantly increased (> 40%) (Figure 5B and 5C). At higher concentrations of curcumin, however, Sal failed to maintain cell viability (Figure 5A). Interestingly, the caspase inhibitor Q-VD-OPh did not reduce curcumin-induced cell death (Figure 5B).

Antioxidants and the MEK1/2 inhibitor U0126 prevent curcumin-induced cell death

Antioxidants such as N-acetylcysteine (NAC), reduced glutathione (GSH) and sodium hydrogen sulfide (NaHS) are substances with known cytoprotective properties and potent scavengers of reactive oxygen species (ROS). To study whether curcumin-induced cytotoxicity might be diminished by these substances, MRC-5 cells were loaded for 1 h with NAC, GSH (each 1 mM) or NaHS (0.1 mM) before being treated for 6 h with curcumin (25 μM). In comparison to curcumin-treated cells, NAC reduced LDH activity by about 80%, GSH and NaHS by about 50–60%, suggesting that antioxidants had strong beneficial effects on cell viability (Figure 6). The MEK1/2

inhibitor U0126, which blocks activation of ERK1/2, also reduced curcumin-induced cell death (~ 50%) (Figure 6).

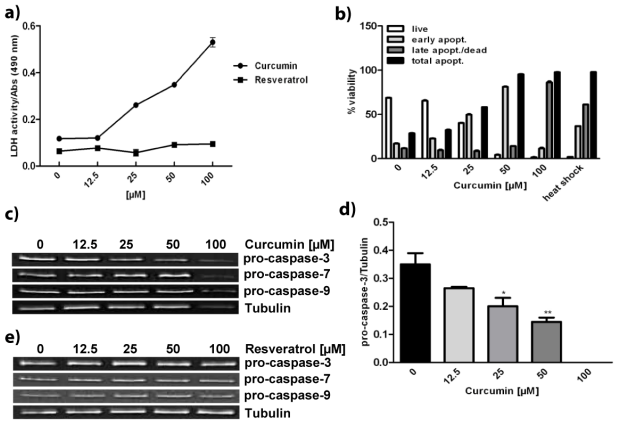

Figure 4: Cytotoxic and pro-apoptotic effects of curcumin and resveratrol on MRC-5 cells.(A) Cells were treated for 6 h with indicated concentrations of curcumin or resveratrol and LDH activity was then measured in cell culture supernatants. (B) MRC-5 cells were treated for 6 h with indicated concentrations of curcumin and cells were stained with Annexin-V/7-AAD to differentiate between cells being alive or in the early or late apoptotic stage. (C, E) MRC-5 cells were treated for 1 h with indicated concentrations of curcumin or resveratrol and activation of caspase-3/-7/-9 was analyzed by Western blot. (D) Levels of pro-caspase-3 in curcumin-treated cells were semi-quantitatively determined by densitometry. Data are representative of 2 experiments (*p<0.05; **p<0.01).

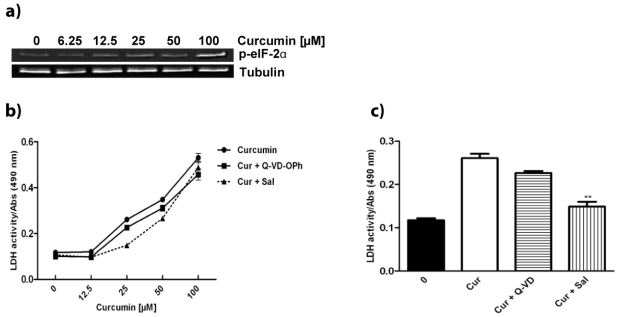

Figure 5: Curcumin induces ER stress in MRC-5 cells. Cells were treated for 30 min with indicated concentrations of curcumin. Total cell proteins were subjected to SDS-PAGE and blotted onto nitrocellulose membranes. (A) Phospho-eIF-2α (p-eIF-2α) was detected with a specific antibody against ser51. (B, C) Cells were treated for 6 h with indicated concentrations of curcumin in the absence or presence of Salubrinal (50 µM) or Q-VD-OPh (25 µM) and LDH activity was then monitored in cell culture supernatants.

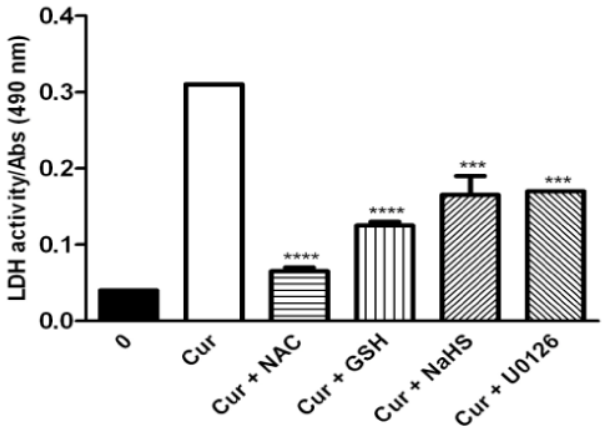

Figure 6: NAC, GSH, NaHS and U0126 reduce the cytotoxic effects of curcumin in MRC-5 cells. Cells were loaded for 1 h with NAC, GSH (each 1 mM), NaHS (0.1 mM) or U0126 (10 µM) and were then incubated with 25 µM curcumin for another 6 h. LDH activity was measured in cell culture supernatants. Data from 2 independent experiments are presented as mean ± SEM (***p<0.001; ****p<0.0001).

Discussion

Our data demonstrate that the phytochemicals curcumin and resveratrol had pronounced anti-inflammatory activities in the human lung fibroblast cell line MRC-5. Both compounds diminished IL-1β and PMA-induced IL-6 gene expression in a concentration-dependent manner. In the NF-κB signaling cascade, NF-κB once activated immediately initiates the transcription of IL-6 mRNA and many other pro-inflammatory genes (TNF-α, IL8, COX-2, etc.). Our data show that curcumin at 12.5 µM almost completely blocked IL-6 expression in MRC-5 cells. In strong contrast, Western blot analysis revealed that curcumin suppressed phosphorylation of NF-κB only at a concentration of 100 µM. It has been shown that besides the phosphorylation of the NF-κB subunit p^{65} (RelA) at serine 276/536, acetylation at lysine 218, 221, and 310 differentially regulates the DNA binding activity of RelA along with its assembly with IκBα, and transcriptional activity [15]. The acetyltransferases p300 and CBP appear to play a major role in the *in vivo* acetylation of RelA [16]. Balasubramanyam et al. has been reported that curcumin repressed acetylation of histone/nonhistone proteins and histone acetyltransferase-dependent chromatin transcription [17]. Therefore, we suggest that the inhibitory effects of curcumin on IL-6 expression may be due to its ability to prevent acetylation of RelA but in further experiments we will clarify this hypothesis. In contrast to curcumin, the inhibitory effect of resveratrol on IL-6 expression was significantly lower (~10 fold less). Western blot data demonstrate that resveratrol blocked neither RelA phosphorylation nor degradation of IκBα. Recently, it has been reported that activation of Sirtuin1 (Sirt1) by resveratrol inhibited TNF-α-induced inflammation in mouse embryonic fibroblasts [18]. Sirt1, a mammalian homolog of Sir2, is a NAD^{+} -dependent class III histone deacetylase. Sirt1 is involved in a variety of physiological and pathophysiological processes, such as cell growth, inflammation and modulation of cancer cell metabolism

[19,20]. Knockout of Sirt1 led to an increase in cytokine expression, whereas Sirt1 activation inhibited production of TNF-α, monocyte chemoattractant protein 1 (MCP-1) and IL-8 [21]. Suppression of pro-inflammatory cytokines production by Sirt1 is highly related to its negative regulation of NF-κB activity via the deacetylation of the RelA subunit at lysine 310 [22]. Based on these findings we conclude that curcumin and resveratrol are both anti-inflammatory compounds but may act by different molecular mechanisms. On the one hand, curcumin may prevent acetylation of RelA at lysine 310 thereby blocking acetyltransferase p300 and CBP, while resveratrol, on the other hand, may induce deacetylation of RelA by activation of Sirt1.

Caspases play a key role in programmed cell death mediated by various apoptotic stimuli [23]. Curcumin was found to act as an antioxidant at low dose, whereas at higher concentrations its prooxidant properties prevailed, a virtue that may be of importance in cell apoptosis [24]. We demonstrated that curcumin, even at relatively low concentrations (25 μM) had negative effects on cell viability. This finding stays in contrast to the results of another group which has been demonstrated that NIH3T3 mouse fibroblasts and HEK-293 cells were largely resistant to curcumin treatment and displayed minimal cell death toxicity at 100 μM suggesting different sensitivities of distinct cell types to curcumin [25]. Additionally, our data show that curcumin induced cell death through apoptosis via activation of caspase-3. Apoptosis is often associated with the induction of endoplasmic reticulum (ER) stress [26]. Phosphorylation of the alpha unit of the eukaryotic initiation factor-2 (eIF-2α) is a marker for ER stress [27]. Our findings demonstrate that curcumin besides its capability to induce apoptosis also induced phosphorylation of eIF-2α. Inhibition of dephosphorylation eIF-2α by Salubrinal significantly protected cells from curcumin-induced cell damage. Furthermore, antioxidants such as NAC, GSH and NaHS also increased the survival rate of curcumin-treated cells. It has recently been shown that in the leukemic monocyte cell line THP-1, curcumin-induced apoptosis was induced via phosphorylation of ERK1/2 [28]. We showed that in MRC-5 cells, phosphorylation of ERK1/2 was induced at 50 μM curcumin, a concentration which definitely led to programmed cell death. Therefore, we suggest that the underlying mechanisms for the initiation of apoptosis in THP-1 and MRC-5 cells might be similar. In contrast to curcumin, resveratrol had no negative influence on cell viability and did not induce apoptosis.

Conclusion

Recently, we reported about the anti-inflammatory and pro-apoptotic effects of curcumin on human synovial fibroblasts derived from rheumatoid arthritis patients [29,30]. In the present study, we investigated the effects of curcumin and resveratrol on the human lung fibroblast cell line MRC-5 and data demonstrated that the effects of curcumin were very similar. In accordance with our results, Smith et al. reported that curcumin inhibited fibrosis-related effects in idiopathic pulmonary fibrosis (IPF), a progressive and fatal lung disease for which not therapy has been identified. Curcumin reduced lung fibroblast cell proliferation and inhibited collagen secretion by IPF fibroblasts [31]. The potential use of curcumin and resveratrol as a therapeutic option is very limited through its low bioavailability. Therefore, in cooperation with the Department of Nanobiotechnology (University of Natural Resources and Life Sciences, Vienna, Austria) we plan to evaluate the effects of so called "CurcuEmulsomes" in our *in vitro* assays. CurcuEmulsomes are spherical solid nanoparticles and increase the bioavailability of curcumin by up to 10.000 fold as recently reported [31,32]. CurcuEmulsomes might be a valuable tool to study the beneficial effects of curcumin in various therapeutic applications *in vivo.*

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