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 p38 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 proinflammatory activity [6], triggers accumulation of cytosolic Ca2+ [7], and disrupts protein disulfide bond formation [8], all of which initiate ER stress response.

Resveratrol (trans-3,4,5-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% CO2 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 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 MuseTM 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 MuseTM 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 (pH 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.
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 p65 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).

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).

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 completely blocked curcumin-induced cell death (Figure 5B).
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 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 p65 (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.
Therefore, in cooperation with the Department of Nanobiotechnology from curcumin-induced cell damage. Furthermore, antioxidants such on the other hand, may induce deacetylation of RelA by activation of Sirt1. 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 proxidant 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 fibroblasts of patients with rheumatoid arthritis. Int J Mol Med 20: 365-372.


References


