Caffeic Acid Phenethyl Ester Inhibits the Proliferation of HEp2 Cells by Regulating Stat3/Plk1 Pathway and Inducing S Phase Arrest

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Summary

Caffeic acid phenethyl ester (CAPE), an active polyphenolic component of honeybee propolis, has been demonstrated to have many medicinal properties. However, the antitumor effect and mechanism of CAPE on laryngeal carcinoma cells have not been examined. In this study, we treated HEp2 cells with various concentration of CAPE, and the results showed that CAPE can reduce the viability of HEp2 cells with IC₅₀ values of 23.8 ± 0.7 μM for 72 h. Meanwhile, CAPE significantly inhibited activation of Stat3 in a concentration dependent manner in HEp2 cells and regulated the expression and transcription of Plk1. AG490, a specific Stat3 inhibitor, not only inhibited the activation and expression of Stat3, but also inhibited the expression of Plk1 in HEp2 cells, so Stat3 was probably involved in the regulation of Plk1 in HEp2 cells. In addition, treatment of CAPE leaded to a blockage of cell cycle in S phase in HEp2 cells. Therefore, CAPE inhibited the proliferation of HEp2 Cells probably by regulating Stat3/Plk1 pathway and inducing S phase arrest.

Keywords

Caffeic acid phenethyl ester (CAPE), laryngeal carcinoma, Stat3/Plk1 pathway
1. Introduction

Laryngeal carcinoma as a malignant carcinoma of head and neck region is the 11th most common cancer in people all over the world, which also is the most common head and neck malignancy in northeast China\textsuperscript{1,2}). The surgical treatment, chemotherapy and radiotherapy make a significant increase in survival with cure rates of up to 80\% to 90\% for patients at early stage, however, the survival decreases to as low as 40\% in patients at late stage\textsuperscript{1,3}). Meanwhile, it remains unsatisfactory for distant metastasis and relapse\textsuperscript{4,5}). Therefore, more efforts need to develop new approaches and strategies for treatment of laryngeal carcinoma.

Stat3 as a transcription factor is phosphorylated to regulate transcription of target genes in response to cytokines and growth factors\textsuperscript{6}). Aberrant activation of Stat3 promotes proliferation and oncogenesis by regulating transcription of various genes\textsuperscript{7}). Plk1 is a key regulator of cell division\textsuperscript{8}). Deregulation of Plk1 has been demonstrated to lead to tumorigenesis\textsuperscript{9}). Previous studies have shown that Stat3 and Plk1 control each other's transcription in a positive feedback loop that leads to tumor development\textsuperscript{10}). Aberrant activation of Stat3 and frequently overexpression of Plk1 have been found in various tumors, including laryngeal carcinoma\textsuperscript{11,12}). Therefore, Stat3/Plk1 pathway may be a potential target for laryngeal carcinoma therapy.

Caffeic acid phenethyl ester (CAPE), an active polyphenolic component of propolis, has been reported to exhibit various bioactive properties such as antitumor\textsuperscript{13,14}) and neuroprotection\textsuperscript{15,16}). CAPE suppresses proliferation of tumor cells through various mechanisms including inducing apoptosis and inhibiting the angiogenesis\textsuperscript{17,18}). However, the antitumor effect and mechanism of CAPE on laryngeal carcinoma cells have not been examined. In this report, we
explored antitumor effects and underlying mechanisms of CAPE against HEp2 in vitro.

2. Materials and Methods

2.1 Cell line and culture

The cell line (HEp2) was purchased from Boster Biological Technology co. ltd (Wuhan, China). The cells were cultured in RPMI 1640 with 10% fetal bovine serum (FBS; PAN) and 1% penicillin-streptomycin antibiotics (Hyclone) in a humidified incubator with 5% CO2 at 37°C.

2.2 Anti-proliferation activity assay

Cell viability was detected using the MTS assay (Promega) according to the manufacturer’s instructions. Briefly, HEp2 cells were plated in 96-well plates at 4000 cells/well. After incubated for 12 h, the cells were exposed to CAPE (J&K Scientific) or AG490 (Beyotime Biotechnology) at various concentrations. After 24 h, 48 h or 72h, 20 µL MTS was added in per well and incubated at 37°C for 3 h. Absorbance of each well was detected by a microplate reader at a wavelength of 490 nm.

2.3 Colony formation assay

To assess the clonogenic capacity of cells in vitro, 500 cells were plated in six-well culture plates. Following 24 h incubation, the cells were treated with various concentrations of CAPE for 24 h. After being washed with PBS, the cells were allowed to grow for 10 days to form colonies, and then fixed with methanol and stained with crystal violet.

2.4 Western blot analysis

After treatment, western blotting was performed according to the standard protocol. Briefly,
total protein was extracted from cells after 24 h of treatment CAPE or 2.5 h of treatment of AG490. Equal amounts of protein lysates were separated on SDS-PAGE gels and then transferred to PVDF membranes. The blots were blocked and probed with primary antibodies against Stat3 (12640, Cell Signaling Technology), phospho-Stat3 (Tyr 705) (9145, Cell Signaling Technology) and Plk1 (sc5585, Santa Cruz). β-actin (PM053, MBL) was used as a loading control. Then the signals were detected by enhanced chemiluminescence detection reagent (Applygen).

2.5 Cell cycle analysis

Cell cycle was tested by flow cytometry. Cells were harvested after 12 h of treatment with various concentrations of CAPE and fixed in ice-cold ethanol at -4°C overnight. The cells were centrifuged to remove the fixing solution. The RNase was added and incubated 30 min, and cells were stained with propidium iodide for 15 min in dark conditions at room temperature. The cells were then analyzed by flow cytometry (FACSVersetm, BD).

2.6 qRT-PCR analysis

Cells were harvested after 24 h of treatment with CAPE and total RNA were isolated using Trizol reagent (Invitrogen). The cDNA was gained with the Transcript Reverse Transcription System (Promega). The reverse-transcription products were amplified by an ABI 7300 Detection System (Applied Biosystems) using the SYBR Green PCR Master Mix (Roche) and the primers. The primers of genes are shown in Table 1. The value gained for each gene was normalized to that of the gene encoding GAPDH. The change-in-cycling-threshold ($2^{-\Delta\DeltaCt}$) method was used for calculation of the relative expression level of the genes.
2.7 Statistical analysis

Statistical analysis was performed using the GraphPad Prism 5. The significance of differences was determined by Student's t-test or one-way ANOVA. A value of \( P < 0.05 \) was identified as statistical significance.

3. Results

3.1 CAPE inhibits the proliferation of HEp2

To explore the potential anti-proliferation effect of CAPE to laryngeal carcinoma cells, we first treated HEp2 cells with increasing concentrations of CAPE for 24, 48 and 72 h followed by MTS assay. We observed that CAPE exposure slightly inhibited the HEp2 cells viability at 24 h (Fig. 1a), but exhibited significant inhibitory effects at 48 and 72 h in a concentration dependent manner (Fig. 1b and 1c). The IC\(_{50}\) values of CAPE in HEp2 cells for 72 h were 23.8 ± 0.7 μM. Gefitinib was used to be a positive control, which had good efficacy in the treatment of squamous cell carcinoma of the head and neck in clinical studies, and showed obvious anti-proliferative effect on HEp2 cells (Fig. 1d).\(^9\) In addition, CAPE exposure inhibited colony formation of HEp2 cells in a concentration dependent manner (Fig. 1e). HEp2 cell growth states exposed to 0, 10, 20, 40 and 80 μM CAPE for 48 h were shown in Fig. 1f. These results suggest that CAPE has the potential to inhibit the proliferation of laryngeal carcinoma cells.

3.2 Stat3 is involved in the regulation of Plk1 in HEp2 cells

Studies demonstrated that Stat3 directly activated Plk1 transcription and Plk1 potentiated Stat3 expression in some human tumors, such as esophageal squamous cell carcinoma (ESCC).
cells\textsuperscript{10} and lung adenocarcinoma epithelial cells\textsuperscript{20}. However, the relationship between Stat3 and Plk1 in laryngeal carcinoma cells is unclear. To investigate the involvement of Stat3 on Plk1, we treated HEp2 cells with AG490\textsuperscript{21}, a specific inhibitor of Stat3, at 10 µM for 2.5 h. The results showed that AG490 not only inhibited the activation and expression of Stat3, but also inhibited the expression of Plk1 (Fig. 2a and 2b). Furthermore, we treated HEp2 cells with increasing concentrations of AG490 for 48 h followed by MTS assay. We observed that AG490 exposure inhibited the proliferation of HEp2 cells (Fig. 2c). Therefore, we speculated that Stat3 was involved in the regulation of Plk1 in HEp2 cells and the Stat3/Plk1 pathway played an important role in proliferation of HEp2 cells.

3.3 CAPE regulates Stat3/Plk1 pathway in HEp2 cells

To investigate whether CAPE could regulate Stat3/Plk1 pathway in human laryngeal carcinoma cells, we respectively treated HEp2 cells with 10 µM, 20 µM, 40 µM and 80 µM CAPE for 24 h. As a result, expressions of Stat3 and p-Stat3 (Y705) were significantly reduced in a concentration dependent manner (Fig. 3a and 3b). Meanwhile, Plk1 as a directly activated gene by Stat3 was significantly rose in 20 µM CAPE exposure and significantly lowered in high concentration of CAPE exposure. Furthermore, we investigated the transcriptional level of Stat3 and Plk1 in CAPE treated HEp2 cells, we preformed qRT-PCR at 10 µM, 20 µM, 40 µM and 80 µM CAPE exposure (Fig. 3c and 3d). The transcriptional level of Stat3 and Plk1 was consistent with the expression level. Taken together, these results demonstrated that CAPE inhibited cell proliferations in HEp2 cells probably by regulating Stat3/Plk1 pathway.
3.4 CAPE arrested the cell cycle at S phase in HEp2 cells

Plk1 have emerged as critical regulators of cell cycle, so we further detected the effects of CAPE on cell cycle progression. Exposure CAPE to HEp2 cells for 12 h caused an interference with cell cycle distribution. As shown in Fig. 4, treatment of CAPE led to a blockage of cell cycle in S phase. When HEp2 cells were treated with 10 µM of CAPE, compared with control, the percentage of cells in G2/M phase decreased from 33.6% to 18.4%. The percentage of cells in S phase increased form 9.7% in control to 21.2% in 10 µM CAPE treatment. When HEp2 cells were treated with 20 µM CAPE, there was the similar level arrest as 10 µM CAPE treatment. However, the inhibition of Plk1 generally induces G2/M arrest, but not S arrest. Thus, the S phase arrest was not induced by Plk1 inhibition. Stat3 can arrest cell cycle progression through many pathways\(^{22}\), and CAPE may induce cell cycle arrest through other pathway. Anyhow, the S phase arrest is an important factor to inhibit the proliferation of HEp2 cells.

4. Discussion

We present here that CAPE inhibits the proliferation of human laryngeal carcinoma HEp2 cells, which partly attributes to the inhibition effect of Stat3/Plk1 pathway and the S phase arrest.

Previous studies have demonstrated that Stat3 directly activates Plk1 transcription through binding to the PLK1-SIE element in the PLK1 promoter, and Plk1 potentiates Stat3 expression as well in esophageal squamous cell carcinoma cells. Stat3 and Plk1 control each other’s transcription in a positive feedback loop that contributes to the development of
In this report, we found that Stat3 was also involved in the regulation of Plk1 in HEp2 cells, and the Stat3/Plk1 pathway played an important role in proliferation of HEp2 cells. Furthermore, CAPE was found to regulate Stat3/Plk1 pathway in HEp2 cells to inhibit the proliferation of HEp2 cells.

Our results showed that the expression and transcriptional level of Plk1 were increased in a low concentration CAPE treatment in HEp2. Plk1 is overexpressed in a wide spectrum of tumors and is frequently considered as an oncogene, which implies that overexpression of Plk1 could promote tumor development. However, recent studies show that the overexpression of Plk1 prevents the development of Kras-induced and Her2-induced mammary gland tumors by perturbing mitotic progression and cytokinesis. Therefore, we speculated that, on the one hand, inhibiting expression of Plk1 in a high concentration CAPE treatment can generate the therapeutic benefits, on the other hand, Plk1 up-regulation may have tumor-suppressive properties by perturbing mitotic progression and cytokinesis in HEp2 cells. We will further study the mechanism in the follow-up work.

5. Conclusion

Here, we report that CAPE has potent antitumor activity against HEp2 cells by reducing cell proliferation in vitro. We further identify the Stat3/Plk1 signaling pathway as a pivotal molecular target of CAPE in HEp2 cells.

Acknowledgments

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Conflict of interest:

The authors declare no conflict of interest.
References


Figure 1. CAPE inhibits the proliferation of HEp2 cells. a, b, c. HEp2 cells were treated with 0, 10, 20, 40 and 80 μM CAPE for 24 h, 48 h or 72 h. Cell viability was detected with a MTS assay. c. Gefitinib was a positive control and its inhibitory effect for proliferation of HEp2 cells at 0, 5, 10 and 20 μM for 72 h. Data are expressed as the mean ± SD, n = 3. e. Colony formation assay with HEp2 cells exposed to 0, 5, 10, 20, 40 and 80 μM CAPE for 10 days. f. HEp2 cell growth states exposed to 0, 10, 20, 40 and 80 μM CAPE for 48 h.
Figure 2. AG490, a specific inhibitor of Stat3, inhibited the expression of Plk1 and reduced cells viability of HEp2 cells. a. HEp2 cells were treated with 0 and 10 µM AG490 for 2.5 h, then the cell lysates were immunoblotted for the indicated proteins. b. The data were expressed as the intensity of protein bands of the target genes/β-actin relative to the control group. Data are expressed as the mean ± SD, n = 3. c. HEp2 cells were treated with various concentrations of AG490 for 48 h. Cell viability was detected with a MTS assay. Data are expressed as the mean ± SD, n = 3.
Figure 3. CAPE regulated the Stat3/Plk1 pathway in HEp2 cells. **a.** HEp2 cells were treated with 0, 10, 20, 40 and 80 µM CAPE, then the cell lysates were immunoblotted for the indicated proteins. **b.** The data were expressed as the intensity of protein bands of the target genes/β-actin relative to the control group. Data are expressed as the mean ± SD, n = 3. **c, d.** HEp2 cells were treated with 0, 10, 20, 40 and 80 µM CAPE, then gene mRNA levels of STAT3 and Plk1 were examined by qRT-PCR. GAPDH was used as an internal reference. Data are expressed as the mean ± SD, n = 3.
Figure 4. CAPE mainly arrested the cell cycle at S phase in HEp2 cells. a. The HEp2 cells were treated with 0, 10, and 20 µM CAPE for 12 h, then the cell cycle distribution was determined using flow cytometry. b. The data were expressed as the percentage of cells in G1, S and G2 phase. Data are expressed as the mean ± SD, n = 3.
Table 1.

**Nucleotide sequences of the primers used for qRT-PCR**

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<th>Gene</th>
<th>Forward primer (5’-3’)</th>
<th>Reverse primer (5’-3’)</th>
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