Basic Research Paper

Promotive effects of epoxyeicosatrienoic acids (EETs) on the proliferation of tumor cells

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Background and Objective: Epoxyeicosatrienoic acids (EETs) are generated from arachidonic acid by cytochrome P450 (CYP). Previous studies have revealed a very strong and selective expression of CYP epoxygenase in human cancer tissues, but almost none in adjacent normal tissues. This study investigates the promotive effect of EETs on the proliferation of tumor cells and the possible mechanisms behind it. Methods: Four tumor cell lines, Tca-8113, A549, Ncl-H446 and HepG2 were treated with different concentrations of EETs (8,9-EET, 11,12-EET and 14,15-EET) for 12, 24, 48 and 72 h, respectively. Cell proliferation was measured using the MTT assay. The effect of exogenous EETs on the cell cycle of Tca-8113 cells was assessed by flow cytometry. Signal transduction inhibitors of PI3K (LY294002), MAPKK (PD98059), MAPK (apigenin) and PKC (H7) were used to block EETs-induced cell proliferation. Expressions of the total protein and phosphorylated ERK1/2 and Akt were determined by western blot. Results: EETs promoted the proliferation of tumor cells compared with the control and the vehicle group in a dose- and time-dependent manner (p < 0.01). The incubation of tumor cells with EETs markedly increased the cell number at S/G2-M phase. The percentages of Tca-8113 cells at S and G2-M phases were (49.7 ± 7.5%) vs. (17.2 ± 9.7%) (p < 0.01) and (21.0 ± 5.3%) vs. (4.9 ± 7.3%), respectively (p < 0.01), with and without the treatment of 11,12-EET. EETs incubation significantly enhanced phosphorylation of MARK as well as PI3K/Akt in tumor cells. LY294002, PD98059, apigenin and H7 reduced the stimulative effect of EETs on cell proliferation. Conclusion: EETs possess the promotive effect on proliferation of tumor cells via activation of MAPK and PI3K/Akt signal pathways.

Epoxyeicosatrienoic acid (EETs) is synthesized from arachidonic acid (AA) by the cytochrome P-450 epoxygenase (CYP). Vast amounts of research demonstrates that EETs have multiple important biological functions, the primary one of which is to act as an endothelium-dependent hyperpolarizing factor (EDHF) for dilating the blood vessels of the heart and kidneys, as well as effecting anti-inflammation, angiogenesis and apoptotic inhibition. Current understanding reveals that EETs also have a promotional effect on the proliferation of endothelium cells in the kidneys and large arteries, and is closely related to the signal transduction.

Current discoveries also find that CYP epoxygenase and its metabolic products are possibly closely related to the incidence and progression of tumors. This discovery has led us to further investigate the effects and mechanisms of EETs in important biological processes in tumors such as proliferation, apoptosis and metastasis. In this research, conducted for the purpose of understanding the influence of EETs on the proliferation of tumor cells, we directly and primarily implanted EETs, which was synthesized from the families of CYP2C and 2j, on four different tumor cells to observe its effect and to investigate its signal transduction mechanism.

Materials and Methods

Primary agents. We purchased 8,9-EET, 11,12-EET, 14,15-EET, PD98059, apigenin, LY294002, H7, 17-ODYA, [3-(4,5-dimethylthiazol)-2,5-diphenyltetrazoliumromide, MTT], propidium iodide (PI), and RNase A from US Sigma Company, and EETs was dissolved in anhydrous ethanol fetal bovine serum, DMEM culture media, and pepsin, which were from the GIBCO Company (Germany). Anti-ERK1/2 and anti-phosphorylation ERK1/2 antibodies were purchased from New England Biolabs (NEB). Anti-PI3K antibodies, anti-Akt antibodies, anti-phospho-
The influence of extrinsic EETs on the proliferation of tumor cells. The influence of different concentrations of EETs on the proliferation of tumor cells was measured by MTT method. Tca-8113 cells in logarithmic growth were selected and digested by 0.25% trypsin, while its cellular concentration was adjusted by DMEM into 1 x 10^5/L of singular cell suspension. We took 100 μL and inoculated it onto a 96-well cell culture plate, before incubating it for 12 hours at 37°C. Afterward, the culture media were replaced with 8,9-EET, 11,12-EET and 14,15-EET under Argon (because EETs can easily be oxidized in air and its half-life period is shorter, the addition must be completed under Argon and the step repeated once every six hours) to make the final concentrations as 50, 100 and 200 nmol/L. For each concentration three parallel wells were set and the interfering drugs were not added into the control group. After 48 hours of incubation, the culture media was discarded and 20 μL MTT of 10 mg/mL was added into each well and it was continued incubating for another four hours at 37°C. Supernatant was discarded and 200 μL dimethyl sulfoxide was added into each well. A warm water bath was given for ten minutes. Using the immunoassay instrument, which was set at 490 nm wavelength, the absorption value (A_{490 \text{ nm}}) of each well was measured. Next, the equation below was used to calculate the cell proliferation rate:

\[
\text{Proliferation rate} = \frac{(A_{\text{experiment}} - A_{\text{control}})}{A_{\text{control}}} \times 100\%.
\]

The study of the signal transduction pathway for promoting the proliferation of tumor cells by EETs. We started from the classical signal transduction pathways for cell proliferation, such as MAPK, PI3K/Akt and PKC, to study the promotional effect of EETs on the signal transduction pathway for tumor proliferation.

The levels of phosphorylation and the total expression levels of signal transduction molecules were measured by western blot. Tca-8113 tumor cells were inoculated onto 6-well plate (approximately 3 x 10^4 cell counts per well) and held for two days for 80% of them to fuse. Afterward, vehicle (anhydrous ethanol), 17-ODYA (100 μmol/L), while 8,9-EET (100 nmol/L), 11,12-EET (100 nmol/L), and 14,15-EET (100 nmol/L) were respectively added into the experimental groups. Next, after 24 hours of incubation at 37°C, MTT staining was performed. The A_{490 \text{ nm}} values of each group were calculated, and using the equation in section 1.3.1, the cell proliferation rate was calculated.
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Results

The influence of different concentrations of EETs on tumor proliferation. In comparison to the control group, different concentrations of EETs all had a significant promotional effect on tumor proliferation, and as the concentration of EETs increased, such effect became more prominent. By contrasting three different EET effects, 11,12-EET and 14,15-EET had more significant promotional effect on tumor (p < 0.01), while 8,9-EET was relatively weaker (p < 0.05) (Fig. 1). This result suggests that the effect of EETs on tumor proliferation is concentration-dependent and its strength affected by the chemical structure of EETs.

The influence of different acting times of EETs on tumor proliferation. Three EETs were still promotional on tumor proliferation at different time periods (p < 0.05) and as the acting time of EETs elongated, the promotional effect became more prominent. Also, the proliferation rate of 8,9-EET was significantly lower than 11,12-EET and 14,15-EET (p < 0.05). At the same time, from the time-effect curve of 11,12-EET, the promotional effect on tumor growth by EET became stronger as the acting time of 11,12-EET elongated and the dosage increased. Plus, the high concentrations of 11,12-EET (100 nmol/L and 200 nmol/L) could greatly reduce the time for logarithmic growth of tumor cells. This result suggests that the promotional effect of EETs on tumor proliferation is time-dependent and its strength is affected by the chemical structure of EETs (Figs. 2 and 3).

The influence of extrinsic EETs on tumor proliferation. For further proof of the promotional effect of EETs on tumor cells we only selected 8,9-EET, 11,12-EET and 14,15-EET to act on four tumor cell lines from different organs. The proliferation status was measured by MTT method. Experimental results showed that after the interference on the four types of tumor cells by three kinds of EETs, the proliferation levels were much higher than that of the control group; it was more apparent in 11,12-EET and 14,15-EET (p < 0.01) and weaker in 8,9-EET (p < 0.05). However, there was no significant different in promotional effect of EETs on tumor cells from different organ origin. On the contrary, the interference by 17-ODYA inhibited the activity of CYP epoxygenase in the cell itself, causing the reduction in synthesis of intrinsic EETs and thus the proliferation level was greatly reduced. This had statistical significance in comparison to the control group (p < 0.01) (Fig. 4). These results all showed that EETs had a wider range of promotional effect on tumor proliferation and had no strict selectivity on the tumor cell based on origin.

The influence of extrinsic EETs on cell cycle. After stimulation by extrinsic EETs the ratios of Tca-8113 cells at each stage were different from the control group. The ratios of cells at S-phase and G2/M phase were significantly increased while the cells in G1 phase were relatively reduced. There was statistical significance (p < 0.01) (Table 1).

The influence of signal transduction molecule inhibitors on the promotional effect of extrinsic EETs on tumor proliferation. Two hours after the addition of MAPKK inhibitor PD98059, MAPK inhibitor apigenin and PI3K inhibitor LY294002, all

Statistical analysis. All data were presented in form of average ± standard deviation. SPSS 12.0 software was used for variance analysis and t-test. Statistical significance was p < 0.05.
significantly inhibited EETs from promoting tumor proliferation. This was statistically significant in comparison to the control group (p < 0.01). On the other hand, two hours after addition of PKC inhibitor, H2 did not inhibit EETs and the comparison to the control group had no statistical significance (p > 0.05). This result suggested that EETs possibly promoted tumor proliferation through MAPK and PI3K signal transduction pathways, while the PKC pathway was not involved (Fig. 5).

The influence of extrinsic EETs on the MAPK and PI3K/Akt signal transduction pathways. The tumor cell proteins, which were stimulated by extrinsic EETs, were extracted and the phosphorylation level and expression levels of total proteins of MAPK and PI3K/Akt were measured by western blot. Experimental results showed that after stimulation of tumor cells by three types of EETs the phosphorylation levels of MAPK and Akt and the protein expression of PI3K were greatly upregulated. This was statistically significant in comparison to the control group (p < 0.05). The expressions of total MAPK and Akt were not changed. Therefore, we believe that extrinsic EETs achieves tumor proliferation through the activation of MAPK and PI3K/Akt pathways (Fig. 6).

Discussion

Early discovery of the influence of EETs on the proliferation and signal transduction in cells was made in endotheliocytes of the kidneys.12 Later, in an experiment involving regular pulling test of the coronary artery, it was found that EETs is a type of EDHF and it was proven that EETs activated a series of kinases, the functions of which are closely related to the proliferation of endothelium cells.15 This study, in order to further ensure the effect and mechanism of EETs on the malignant proliferation of these tumor cells, chose to study four tumor cell lines from different origins (Tca-8113, A549, Ncl-H446 and HepG2). This study, on the influence of extrinsic EETs on cell proliferation, discovered that EETs does have significantly promote tumor growth.

Arachidonic acid (AA) is metabolized into EETs by cytochrome epoxygenase. Because of the difference in position of the alkyl chain, the compounds were named 5,6-EET, 8,9-EET, 11,12-EET and 14,15-EET, respectively, and according to their chemical structures,16,17 in which 5,6-EET has poor stability.12 Therefore, we only chose 8,9-EET, 11,12-EET and 14,15-EET for experiment in this study. First we measured the quantity-effect curves and the time-effect curves of tumor proliferation in 8,9-EET, 11,12-EET and 14,15-EET. The results showed that the promotional effect on proliferation was both time-dependent and concentration-dependent. They also showed that different EETs have different promotional effects, regardless of whether they are shown in a quantity-effect curve or a time-effect curve. The promotional effect of 8,9-EET was significantly weaker than those of 11,12-EET and 14,15-EET. This suggests that the promotional effect of EETs is influenced by its chemical structure. Later, flow cytometry was used to analyze the influence of EETs on Tca-8113 proliferation cycle. Test results, in comparison to the control group, demonstrated that Tca-8113 in S-phase and G2/M-phase, after stimulation by EETs was greatly increased, while cells in G1-phase were reduced. It suggested that cells in the active period of DNA

![Figure 3. Effects of different concentrations of 11,12-EETs on the proliferative activity of Tca-8113 cells at different time courses. Data are expressed as mean ± SD of three independent experiments. (A) p < 0.05, (B) p < 0.01 vs. vehicle group.](image)

![Figure 4. Effects of 8,9-EET, 11,12-EET and 14,15-EET on the proliferation of four tumor cell lines. Cell were cultured with 100 μmol/L 17-ODYA, 100 nmol/L of 8,9-EET, 11,12-EET and 14,15-EET for 24 h. Data are expressed as mean ± SD of three independent experiments. (A) p < 0.05, (B) p < 0.01 vs. vehicle group, (C) p < 0.05 vs. the 17-ODYA group.](image)

<table>
<thead>
<tr>
<th>Group</th>
<th>Cell cycle distribution (%)</th>
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<th>Cell cycle distribution (%)</th>
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<tr>
<td></td>
<td>G0/G1</td>
<td>S</td>
<td>G2/M</td>
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<tr>
<td>Control</td>
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<td>17.2±9.7</td>
<td>4.9±7.3</td>
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<tr>
<td>8,9-EET</td>
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<tr>
<td>11,12-EET</td>
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<td>21.0±5.3*</td>
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<tr>
<td>14,15-EET</td>
<td>34.6±9.1</td>
<td>47.8±10.2</td>
<td>17.7±11.3*</td>
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*Data are expressed as mean ± SD of three independent experiments. p < 0.05, p < 0.01 vs. the control group.
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Recent research has discovered that the in vivo expression of cytochrome epoxygenase is selective of cell and tissue and thus the concentration of produced EETs also vary among different tissues or cells, such as in the case of CYP2J2, which is widely distributed in the heart, liver, lungs, kidneys, intestine, pancreas and blood vessels, especially in the heart and vascular endothelium cells.\(^{18,19}\) Also, its expression strength varies among different tumor cells.\(^{13}\) Therefore, in order to prove the wide range of promotional effect of EETs on tumor cells, we selected four tumor cell lines of different origins (Tca-8113, A549, Ncl-H446 and HepG2) to study the influence of EETs on tumor proliferation. Experimental results proved that EETs was indeed promotional on all four tumor cell lines and this effect showed no statistical difference across the tumor cells. This suggested that EETs was indeed promotional to various tumors and no selectivity of cell origin was seen.

When we investigated the mechanism of EETs on promoting tumor proliferation, we found that the PI3K and MAPK inhibitors can greatly prevent the effect of EETs while PKC inhibitors did not affect it. This suggests that EETs achieve promoted cell proliferation through PI3K and MAPK pathways and that PKC pathways are not involved. On this basis, the phosphorylation levels of PI3K and ERK1/2 were greatly upregulated after extrinsic EETs stimulated tumor cells. The expressions of total Akt and ERK1/2 showed no change. This further proved that PI3K and MAPK pathways participated in promoting tumor proliferation with EETs. There are reports that the activity of MAPK kinase, which is activated by EETs, could be inhibited by CYP inhibitors and specific antisense CYP2C oligonucleotide. However, in endothelium cells pre-treated by 11,12-EET and the hyper-expression of CYP2C8, kinase activity was enhanced.\(^{15}\) Further analysis on the mechanism of EETs in promoting tumor proliferation revealed that the CYP epoxygenase pathway of AA could transcribe epidermal growth factor receptor (EGFR).\(^{20}\) The EET-mediated EGFR activation could feedback and activate Akt kinase for enhanced expression of Cyclin D1. There was report that these four members of the synthesis were increased, which further proved the promotional effect of EETs on tumor proliferation.
EETs could induce phosphorylation of Akt and proliferation of endothelial cells in rats, but only the proliferating effect mediated by 5,6-EET and 14,15-EET was sensitive to PI3K inhibitors, while 8,9-EET and 11,12-EET seemingly relied on the activation of P38MAP kinase.21 In the endothelial cells of large fetal bovine artery, the proliferations mediated by 8,9-EET, 11,12-EET and 14,15-EET could be minimized by MEK, ERK and PI3K inhibitors.22 The function of EETs in the proliferation of endothelial cells is clear, but its function in the vascular smooth muscle cells remains unclear. For example, it has been reported that 14,15-EET was not effective in the proliferation of vascular smooth muscle cells,23 and there was a contrary report that 14,15-EET could enhance the proliferation of smooth muscle cells, mediated by growth factors of platelet-origin.24 These experimental results showed that different EETs relied on different signal transductions to promote tumor proliferation. This study only selected Tca-8113 cell line for investigating the signal transduction mechanism of EETs in promoting tumor growth and different tumor cells might not produce the same results. This requires further study.

Overall, this study further proves that EETs has a significant promotional effect on tumor growth, and it initially investigates its mechanism. This has great significance in understanding the incidence and progression of tumors and provides new research strategies for the prevention and treatment of tumors in the future.

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References