p-HEPA-EDA, a phenolic compound of virgin olive oil, activates AMP-activated protein kinase to inhibit carcinogenesis

Prem Khanal, Won-Keun Oh, Hyo Jeong Yun, Gwang Mo Namgong, Sang-Gun Ahn1, Seong-Min Kwon1, Hoo-Kyun Choi* and Hong Seok Choi†

BK21 Project Team, College of Pharmacy, Chosun University and 1Department of Pathology, School of Dentistry, Chosun University, Gwangju 501-759, Republic of Korea

*To whom correspondence should be addressed. Tel: +82 62 230 6379; Fax: +82 62 222 5414;
Email: cho@chosun.ac.kr
Correspondence may also be addressed to Hoo-K. Choi.
Email: hgchoi@chosun.ac.kr

Phenolic constituents of virgin olive oil are reported to have antitumor activity. However, the underlying molecular mechanisms and specific target proteins of virgin olive oil remain to be elucidated. Here, we report that dialdehydic form of decarboxymethyl ligstroside aglycone (p-HEPA-EDA), a phenolic compound of virgin olive oil, inhibits tumor promoter-induced cell transformation in JB6 C141 cells and suppress cyclooxygenase-2 (COX-2) and tumor growth induced by adenosine monophosphate-activated protein kinase (AMPK) activation in HT-29 cells. p-HEPA-EDA inhibited 12-O-tetradecanoylphorbol-13-acetate (TPA)-induced phosphorylation of extracellular signal-regulated kinases 1/2 and p90RSK in JB6 C141 cells, resulting in the inhibition of cell proliferation, activator protein-1 transactivation and cell transformation promoted by TPA. Moreover, p-HEPA-EDA strongly inhibited the cell viability and COX-2 expression by activation of AMPK activity in HT-29 cells, resulted from depletion of intracellular adenosine triphosphate. p-HEPA-EDA-induced activation of caspase-3 and poly-adenosine diphosphate-ribose polymerase, phosphorylation of p53 (Ser15) and DNA fragmentation in HT-29 cells, leading to apoptosis. Importantly, p-HEPA-EDA suppressed the colony formation of HT-29 cells in soft agar. In contrast, Compound C, an AMPK inhibitor, and Z-DEVD-FMK, a caspase-3 inhibitor, blocked the p-HEPA-EDA-inhibited colony formation in HT-29 cells. In vivo chorioallantoic membrane assay also showed that p-HEPA-EDA-inhibited tumorigenicity of HT-29 cells. These findings revealed that targeted activation of AMPK and inhibition of COX-2 expression by p-HEPA-EDA contribute to the chemopreventive and chemotherapeutic potential of virgin olive oil against colon cancer cells.

Olive oils possess different phenolic profiles according to the variety of olive and the technical conditions used for production (8,9). Three main classes of phenolic compounds are represented in differing proportions in olive oil: simple phenols (phenolic acids and hydroxylated secoiridoids such as hydroxytyrosol and tyrosol), secoiridoids (oleuropein, the aglycone of ligstrostide and their respective decarboxylated dialdehyde derivatives) and lignans [(1L-1-acetyloxypinoresinol and pinoresinol] (8,9). All three classes possess potent antioxidant properties (1). Lignans, one of the phenolic constituents of virgin olive oil (10), can inhibit skin, breast, colon and lung cancer cell growth (11) and induce apoptosis in colon tumor cells (12). 2-(3, 4-Dihydroxyphenil) ethanol, a phenolic antioxidant derived from olive oil, may induce growth arrest and apoptosis in human colon carcinoma HT-29 cells (13).

Adenosine monophosphate-activated protein kinase (AMPK) is a sensor of cellular energy status, is involved in cancer cell apoptosis (14). Since tumor-suppressor genes such as TSC2 and LKB1 function as upstream kinases of AMPK, AMPK is known as an antiproliferative molecule (15–18). Also, AMPK activation induced by phytochemicals, such as genistein, epigallocatechin gallate and capsaicin, inhibits adipocyte differentiation and induces apoptosis of mature adipocytes (19). AMPK signaling might facilitate growth inhibition and cell death, serving as a new therapeutic target in cancer (20), suggesting that AMPK activation by naturally occurring compounds has potential for cancer therapy. For example, activation of AMPK by natural compounds such as epigallocatechin gallate inhibits cyclooxygenase (COX-2), which is involved in the inflammation process and in colorectal tumorigenesis as well as in breast and skin cancers (21–24).

Here we found that dialdehydic form of decarboxymethyl ligstroside aglycone (p-HEPA-EDA; Oleochantal), a phenolic compound of virgin olive oil, inhibited activator protein-1 (AP-1) activity and cell transformation in JB6 C141 cells. Also, p-HEPA-EDA inhibited cell viability and induced apoptosis in HT-29 colon cancer cells through activation of AMPK and inhibition of COX-2 expression. In addition, p-HEPA-EDA increased the transcriptional activity of p53 by increasing phosphorylation (Ser15) and induced the cleavage of poly-adenosine diphosphate-ribosyl polymerase (PARP) and caspase-3, resulting in DNA fragmentation. The results of this investigation suggested a molecular mechanism that underlies the antitumor activity of p-HEPA-EDA and might partially account for the antitumor effects of virgin olive oil.

Materials and methods

Reagents and antibodies
McCoy’s 5A medium, 1-glutamine, gentamicin and fetal bovine serum (FBS) were purchased from Invitrogen (Carlsbad, CA). Z-DEVD-FMK and Compound C were from EMD Chemicals (Gibbstown, NJ). Polyvinylidene difluoride membrane was obtained from Millipore (Bedford, MA). 3-[4,5-Dimethylthiazol-2-thiazoyl]-2,5-diphenyltetrazolium bromide (MTT) was purchased from Sigma–Aldrich (St Louis, MO). Cell Proliferation ELISA and Brdu (colcemid) were from Roche Applied Science (Indianapolis, IN). Antibodies against phospho-p53 (Ser15), p53, phospho-AMPK, phospho-acetyl-CoA carboxylase (ACC), ACC, cleaved caspase-3, cleaved PARP, caspase-3 and Bcl-2 were from Cell Signaling Technology (Beverly, MA); antibodies against COX-2, PARP, AMPK, goat anti-mouse IgG horseradish peroxidase (HRP), goat anti-rabbit IgG HRP and bovine anti-goat IgG HRP were from Santa Cruz Biotechnology (Santa Cruz, CA). Fixation/Permeabilization Solution (Cytofix/Cytoperm) was from BD Biosciences (San Jose, CA).

Cell culture and transfection
HT-29, SK-BR-3 and HCT-116 cells were obtained from the American Type Culture Collection (Manassas, VA). The cells were cultured in McCoy’s 5A medium supplemented with 10% FBS, glutamine and antibiotics (100 U/ml penicillin and 100 µg/ml streptomycin). JB6 C141 mouse epidermal cells were cultured in modified Eagle’s medium supplemented with 5% FBS. Cells were cultured

Introduction
Olive oil is a primary ingredient in the Mediterranean diet, the consumers of which have a low incidence of coronary heart disease and certain cancers (1). The consumption of olive oil reduces the risk of various types of cancer, including prostate, lung, larynx, ovary, breast and colon cancers (2–7). These accumulated data represent evidence that virgin olive oil, inhibited activator protein-1 (AP-1) activity and cell transformation in JB6 C141 cells. Also, p-HEPA-EDA inhibited cell viability and induced apoptosis in HT-29 colon cancer cells through activation of AMPK and inhibition of COX-2 expression. In addition, p-HEPA-EDA increased the transcriptional activity of p53 by increasing phosphorylation (Ser15) and induced the cleavage of poly-adenosine diphosphate-ribosyl polymerase (PARP) and caspase-3, resulting in DNA fragmentation. The results of this investigation suggested a molecular mechanism that underlies the antitumor activity of p-HEPA-EDA and might partially account for the antitumor effects of virgin olive oil.
cultured at 37°C in humidified air containing 5% CO₂. Dimethyl sulfoxide was used as a vehicle to dissolve the compound and a final concentration of 0.1% dimethyl sulfoxide (vol/vol) was used for each treatment. The silencing of human AMPK (accession number: NM_206907) was performed by transfecting the ON-TARGETplus small interfering RNA (siRNA) SMART pool-specific or non-specific pool-specific siRNA oligonucleotides (Dharmacon, Chicago, IL) using Lipofectamine™ 2000 (Invitrogen).

The MTT assay was performed to check cell viability. In brief, cells (1 × 10⁴) were seeded (100 l/well) into 96-well plates with 100 l of medium per well and cultured for 24 h in 6-well plates. The cells were then starved for 24 h and treated with p-HPEA-EDA for 6 h. The cells were then washed with phosphate-buffered saline (PBS) and fixed with Cytofix/Cytoperm at 4°C for 20 min. Then cells were stained with 50 μl TUNEL solution and incubated at 37°C for 1 h. The cells were washed with PBS twice and the cells were fixed. DNA fragmentation was detected using an Axiovert 200 M fluorescence microscope and quantified with Axio Vision software (Carl Zeiss, Thornwood, NY).

**Detection of apoptosis**

The induction of apoptosis was assessed by TUNEL staining and detected with an **in situ** Cell Death detection Kit (Roche Applied Science) according to manufacturer’s instructions. In short, 2 × 10⁴ cells were cultured for 24 h in 6-well plates. The cells were then starved for 24 h and treated with p-HPEA-EDA for 6 h. The cells were then washed with phosphate-buffered saline (PBS) and fixed with Cytofix/Cytoperm at 4°C for 20 min. Then cells were stained with 50 μl TUNEL solution and incubated at 37°C for 1 h. The cells were washed with PBS twice and the cells were fixed. DNA fragmentation was detected using an Axiovert 200 M fluorescence microscope and quantified with Axio Vision software (Carl Zeiss, Thornwood, NY).

**Reporter gene assays**

The reporter gene assay for firefly luciferase activity was performed using lysates from p53-lac-transfected HT-29 cells treated with p-HPEA-EDA. The reporter gene vector, pRL-SV40 (Promega, Madison, WI), was cotransfected into each cell line and the Renilla luciferase activity generated by this vector was used for normalization to the calculated transfection efficiency. Cell lysates were prepared by first washing the transfected HT-29 cells once in PBS at room temperature. After the PBS was completely removed, 100 μl of passive lysis buffer (Promega) was added to each well, and then cells were incubated at room temperature for 1 h with gentle shaking. The supernatant fraction was used to measure firefly and Renilla luciferase activities. Cell lysates (60 μl from each well) were mixed with 50 μl of luciferase assay II reagent (Promega), and firefly luciferase light emission was measured on a TriStar LB 941 (Berthold Technologies GmbH and Co. KG, Germany). Subsequently, 50 μl of Renilla luciferase substrate (Promega) was added to allow normalization of firefly luciferase data. The pGL3-p53-luciferase promoter and -p21-2300-luciferase promoter were provided by Kwang-Youl Lee. (Chonnam National University).

**Anchorage-independent cell transformation assay**

The inhibition of cell transformation of HT-29 cells by p-HPEA-EDA was tested in a soft agar assay. In brief, 8 × 10⁴ cells/ml were seeded in 1 ml of 0.3% basal medium Eagle agar containing 10% FBS, 2 mM L-glutamine and 25 μg/ml gentamicin with p-HPEA-EDA. The cultures were maintained at 37°C for 2 weeks, and cell colonies were scored using an Axiovert 200 M fluorescence microscope and Axio Vision software (Carl Zeiss).

**Choriointoic membrane assay**

The choriointoic membrane (CAM) assay was performed as described previously (26). Briefly, fertilized chicken eggs were transferred to an egg incubator maintained at 37°C and 50% humidity and allowed to grow for 10 days. The fertilized chick eggs were sterilized and a 1 cm² window was cut, using the false air sac technique, on one side of the egg to expose the CAM. HT-29 cells (2 × 10⁴) were placed on the exposed CAM, and the windows were sealed with transparent tape. The eggs were incubated in a humidified incubator at 37°C for 3 days and treated with normal saline or p-HPEA-EDA (50 μg/ml). The CAMs were examined at 4 days after p-HPEA-EDA treatment. Images were digitally recorded at a magnification of ×15 with an SZ-61 zoom stereo microscope (Olympus, Center Valley, PA). Tumor areas were analyzed with ImageJ digital imaging software (download from the National Institutes of Health website).

**Statistical analysis**

**Statistical calculations** were carried out with Prism 4 for Macintosh software (GraphPad Software, La Jolla, CA). Results are expressed as the mean ± standard error of the mean of triplicate measurements of two independent experiments. Student’s t test was used for statistical analyses; P values < 0.05 were considered to be significant.

**Results**

**p-HPEA-EDA inhibits TPA-induced neoplastic cell transformation in JB6 CL41 cells**

We used the phase partition method described by Christophoridou et al. (25) to extract the dihydroyd form of decarboxymethyl lignoside aglycone (p-HPEA-EDA), a phenolic compound in virgin olive oil that belongs to the phenolic class of secoiridoids. The chemical composition of p-HPEA-EDA used in the present work is listed in
p-HPEA-EDA at concentrations from 0.1–5 μg/ml did not affect cell growth at 24 h after treatment in JB6 Cl41 cells (Figure 1B). One of the most important protein kinases activated by TPA is the mitogen-activated protein kinases (27). Thus, we next examined the effect of p-HPEA-EDA on the extracellular signal-regulated kinases (ERK) signaling pathway and found that p-HPEA-EDA significantly suppressed TPA-induced phosphorylation of ERK1/2 and p90RSK, respectively, in JB6 cells in a dose-dependent manner (Figure 1C). Also, the TPA-induced AP-1 activation response was significantly inhibited by p-HPEA-EDA (Figure 1D), which resulted in inhibition of TPA-promoted cell proliferation (Figure 1E) and neoplastic cell transformation in JB6 Cl41 cells (Figure 1F and G).

Figure 1A. p-HPEA-EDA at concentrations from 0.1–5 μg/ml did not affect cell growth at 24 h after treatment in JB6 Cl41 cells (Figure 1B). One of the most important protein kinases activated by TPA is the mitogen-activated protein kinases (27). Thus, we next examined the effect of p-HPEA-EDA on the extracellular signal-regulated kinases (ERK) signaling pathway and found that p-HPEA-EDA significantly suppressed TPA-induced phosphorylation of ERK1/2 and p90RSK, respectively, in JB6 cells in a dose-dependent manner (Figure 1C). Also, the TPA-induced AP-1 activation response was significantly inhibited by p-HPEA-EDA (Figure 1D), which resulted in inhibition of TPA-promoted cell proliferation (Figure 1E) and neoplastic cell transformation in JB6 Cl41 cells (Figure 1F and G).

Activation of AMPK by p-HPEA-EDA suppresses the expression of COX-2 in HT-29 cells

AMPK activation can induce apoptosis by inhibiting proliferating proteins and inducing apoptotic proteins (28). AMPK plays a crucial role in COX-2 regulation in colon cancer cells (29), which in turn regulates cell proliferation and growth. To investigate the effect of p-HPEA-EDA on AMPK activity in HT-29 cells, we treated the cells with p-HPEA-EDA in both a dose- and a time-dependent manner. p-HPEA-EDA time and dose dependently increased the phosphorylation of AMPK and ACC and reduced COX-2 levels (Figure 2A and B). We further examined whether inhibition or ablation of AMPK affects the reduced COX-2 expression by p-HPEA-EDA, respectively. The results showed that p-HPEA-EDA-inhibited COX-2 expression followed with increased phosphorylation of AMPK, whereas treatment of Compound C, an AMPK inhibitor, (Figure 2C) or transfection of siRNA–AMPK (Figure 2D) increased COX-2 expression even in the p-HPEA-EDA-treated HT-29 cells compared with control cells, respectively. Also, p-HPEA-EDA at 0.1–5 μg/ml inhibited TPA-induced COX-2 expression and induced the phosphorylation of AMPK and its downstream effector, ACC, in JB6 Cl41 cells (supplementary Figure S1 is available at Carcinogenesis Online). Overall, these data indicate that p-HPEA-EDA-suppressed COX-2 expression through the regulation of AMPK activity.
Fig. 2. Effects of \( p \)-HPEA-EDA on AMPK activity and expression of COX-2. (A and B) HT-29 cells were serum starved for 24 h and treated with various doses of \( p \)-HPEA-EDA for 6 h (A) or with 5 \( \mu \)g/ml \( p \)-HPEA-EDA for various times (B). Proteins in whole cell lysates were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and immunblotted with antibodies against phospho-AMPK, phospho-ACC, COX-2 and \( \beta \)-actin, respectively. (C and D) HT-29 cells were serum starved for 24 h, pretreated with 10 \( \mu \)M Compound C for 2 h and then treated with 5 \( \mu \)g/ml \( p \)-HPEA-EDA for 6 h (C). Cells were transfected with siRNA–AMPK, serum starved for 24 h and then treated with 5 \( \mu \)g/ml \( p \)-HPEA-EDA for 6 h (D). Proteins in whole cell lysates were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and immunblotted with antibodies against phospho-AMPK, AMPK, phospho-ACC, ACC, COX-2 and \( \beta \)-actin, respectively.

Fig. 3. Effects of \( p \)-HPEA-EDA on cell viability and intracellular ATP levels. (A) HT-29 cells were seeded and treated with \( p \)-HPEA-EDA or 5-amino-4-imidazolecarboxamide riboside for 24 h, respectively. Cell viability was estimated using MTT assay. The inset on the right side indicates expression levels of AMPK in JB6 Cl41 and HT-29 cells or the comparison of the effects of \( p \)-HPEA-EDA (5 \( \mu \)g/ml) and 5-amino-4-imidazolecarboxamide riboside (1 mM) on the AMPK activation, respectively. Columns, mean of triplicate measurements of two experiments; bars, standard deviation. * \( P < 0.05 \), compared with control cells. (B) JB6 Cl41 cells were transfected with pCMV-myc-AMPK, incubated for 24 h and then treated with 5 \( \mu \)g/ml \( p \)-HPEA-EDA for 24 h. Cell viability was estimated using MTT assay. The inset on the right side indicates overexpression levels of AMPK in JB6 Cl41 cells. Columns, mean of triplicate measurements of two experiments; bars, standard deviation. * \( P < 0.05 \), compared with control cells. (C) JB6 Cl41 and HT-29 cells were seeded and treated or not treated with 5 \( \mu \)g/ml \( p \)-HPEA-EDA for 12 h, respectively. Intracellular ATP levels were measured using ATP colorimetric assay. Columns, mean of triplicate measurements of two experiments; bars, standard deviation. * \( P < 0.05 \), compared with control cells. (D) SK-BR-3 and HCT-116 cells were seeded and treated with \( p \)-HPEA-EDA for 24 h, respectively. Cell viability was estimated using MTT assay. The inset on the right side indicates expression levels of AMPK in cells. Columns, mean of triplicate measurements of two experiments; bars, standard deviation. * \( P < 0.05 \), compared with control cells.

\( p \)-HPEA-EDA inhibits the cell viability of HT-29 cells via ATP depletion

To assess the effect of \( p \)-HPEA-EDA on the cell viability of HT-29 cells, we next used the MTT assay. Total AMPK is highly expressed in HT-29 cells compared with JB6 Cl41 cells and \( p \)-HPEA-EDA and 5-amino-4-imidazolecarboxamide riboside, a potent AMPK activator, decreased the cell viability of HT-29 cells dose dependently (Figure 3A), suggesting that \( p \)-HPEA-EDA might induce apoptosis signaling of HT-29 cells through activation of AMPK. Since \( p \)-HPEA-EDA decreased the cell viability of HT-29 cells, but not JB6 Cl41 cells, we further examined whether its effect on cell viability correlates with AMPK expression levels. The results showed that \( p \)-HPEA-EDA significantly inhibited cell viability in transiently AMPK-overexpressing JB6 Cl41 cells (Figure 3B). We also examined whether \( p \)-HPEA-EDA affect intracellular ATP levels differently in HT-29 cells and JB6 Cl41 cells. \( p \)-HPEA-EDA significantly depletes ATP levels from HT-29 cells compared with untreated control, whereas JB6 Cl41 cells were almost protected against the effects of \( p \)-HPEA-EDA on the ATP depletion (Figure 3C). Interestingly, ATP levels in HT-29 cells are significantly higher than in JB6 Cl41 cells (Figure 3C). To examine the effect of \( p \)-HPEA-EDA on cell viability and its correlation with AMPK expression in other cancer cells, SK-BR-3 breast cancer cells or HCT-116 colon cancer cells treated or not treated with \( p \)-HPEA-EDA, respectively. The results showed that \( p \)-HPEA-EDA significantly inhibits the cell viability of HCT-116 cells, which highly expressed AMPK compared with SK-BR-3 cells (Figure 3D). In contrast, SK-BR-3 cells, which have a low expression level of AMPK compared with HCT-116 cells, did not affect with treatment of \( p \)-HPEA-EDA on cell viability, suggesting that the inhibitory effect of \( p \)-HPEA-EDA on cell viability correlated with the level of AMPK expression in these cells (Figure 3D). In addition, \( p \)-HPEA-EDA markedly induced cleavage of caspase-3 and PARP, respectively in
**p-HPEA-EDA induces apoptosis in HT-29 cells**

Next, we investigated the effect of p-HPEA-EDA on the apoptosis-signaling pathway in HT-29 cells. p-HPEA-EDA decreased Bcl-2 levels and induced cleavage of caspase-3 and PARP at the same dose and time as AMPK activation (Figure 4A and B). However, treatment of Compound C, an AMPK inhibitor, and silencing of AMPK using siRNA–AMPK inhibited the activation of caspase-3 and PARP induced by p-HPEA-EDA, respectively (Figure 4C and D). As shown in Figure 3, p-HPEA-EDA downregulated COX-2 expression levels in HT-29 cells, followed with AMPK activation. Therefore, we evaluated the effects of celecoxib, a highly selective COX-2 inhibitor, on the apoptosis cascade signaling. Celecoxib also increased the activity of caspase-3 and PARP in HT-29 cells (Figure 4E). In addition, p-HPEA-EDA induced DNA fragmentation (Figure 4F), suggesting that p-HPEA-EDA leads to apoptosis by activation of AMPK in HT-29 cells.

**p-HPEA-EDA increases the transcriptional activity of p53 in HT-29 cells**

AMPK activation leads to phosphorylation of p53 (Ser15), which is essential for mediating its effects on p53-dependent cell cycle arrest (30). To investigate the effect of p-HPEA-EDA on p53 activation, we treated HT-29 cells with p-HPEA-EDA in a dose- and time-dependent manner. p-HPEA-EDA treatment dramatically increased the phosphorylation of p53 (Ser15) (Figure 5A and B). However, treatment of Compound C and transfection of siRNA–AMPK inhibited the p53 phosphorylation (Ser15) induced by p-HPEA-EDA, respectively (Figure 5C and D). To further determine whether p-HPEA-EDA regulated p53 transcriptional activity, we next cotransfected cells with the p53-responsive luciferase reporter gene, p53-luc. p-HPEA-EDA dose dependently activated p53-luc activity (Figure 5E). Next, we

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**Fig. 4.** Effects of p-HPEA-EDA on apoptotic signaling pathway in HT-29 cells. (A and B) Cells were serum starved for 24 h and treated with 0, 1, 2 or 5 μg/ml of p-HPEA-EDA for 6 h (A) or with 5 μg/ml of p-HPEA-EDA for various times (B). Proteins in whole cell lysates were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and immunoblotted with antibodies against Bcl-2, cleaved caspase-3, caspase-3, cleaved PARP and PARP, respectively. (C and D) Cells were serum starved for 24 h, pretreated with 10 μM Compound C for 2 h and then treated with 5 μg/ml p-HPEA-EDA for 6 h (C). Cells were transfected with siRNA–AMPK, serum starved for 24 h and then treated with 5 μg/ml p-HPEA-EDA for 6 h (D). Proteins in whole cell lysates were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and immunoblotted with antibodies against phospho-AMPK, AMPK, phospho-ACC, ACC, cleaved caspase-3, caspase-3, cleaved PARP, PARP and β-actin, respectively. (E) Cells were serum starved for 24 h and then treated with 25 μM or 50 μM celecoxib for 6 h. Proteins in whole cell lysates were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and immunoblotted with specific antibodies against each protein, respectively. (F) Cells were serum starved for 24 h and treated or not treated with 5 μg/ml of p-HPEA-EDA for 6 h. Cells were fixed and stained with TUNEL solution.
examined whether \( p \)-HPEA-EDA increases the transcriptional activity of p53 on the promoter of p21WAF1 gene using p21-2300-luciferase reporter. \( p \)-HPEA-EDA activated p21-2300-luc in control siRNA-transfected HT-29 cells, whereas the promoter activity was inhibited by knockdown of p53 in HT-29 cells (Figure 5F). Overall, these data indicate that AMPK activation induced by \( p \)-HPEA-EDA is required for p53 transcriptional activation on the promoter of p21WAF1 gene, which subsequently leads to apoptosis.

\( p \)-HPEA-EDA suppresses tumorigenicity of HT-29 cells in vitro or in vivo

Next, we studied the correlation between AMPK activation and tumorigenicity in HT-29 cells using a soft agar colony formation assay. To investigate whether AMPK activation is responsible for the anti-proliferative effects of \( p \)-HPEA-EDA in HT-29 cells, cells were treated with increasing doses of \( p \)-HPEA-EDA (1, 2, 5 and 10 \( \mu \)g/ml) in a soft agar matrix and incubated at 37\(^\circ\)C in a 5\% CO\(_2\) incubator for 10 days. \( p \)-HPEA-EDA, which significantly induced AMPK, inhibited colony formation of HT-29 cells (Figure 6A and B), as measured by colony number and colony size. These results indicate that \( p \)-HPEA-EDA might inhibit the tumorigenicity of HT-29 cells via activation of the metabolic sensor, AMPK. Next, we further examined the effect of AMPK inhibitor or caspase-3 inhibitor on the \( p \)-HPEA-EDA-inhibited colony formation of HT-29 cells. The results showed that Compound C, an AMPK inhibitor, and Z-DEVD-FMK, a caspase-3 inhibitor, blocked the role of \( p \)-HPEA-EDA to inhibit colony formation of HT-29 cells (Figure 6C and D), suggesting that activation of AMPK and caspase-3 induced by \( p \)-HPEA-EDA is essential for chemotherapeutic potential of virgin olive oil in HT-29 cells. Subsequently, the in vivo a CAM assay with HT-29 cells were further performed. Representative images demonstrate a profound reduction of the tumor areas mediated by \( p \)-HPEA-EDA treatment compared with untreated control group (Figure 6E). For statistical evaluation, sections were digitally recorded and tumor areas were analyzed. In line with our in vitro soft agar assay, the \( p \)-HPEA-EDA treatment significantly inhibited tumorigenicity of HT-29 cells in a CAM of chicken embryos compared with control group (Figure 6F).

Discussion

Compounds that induce apoptosis may have chemotherapeutic value since the imbalance of proliferation, apoptosis and differentiation is the main characteristic of cancer cells. Many vegetables in the Mediterranean diet contain substances with anticancer properties (31). The phenolic compounds present in olive oil have anticancer effects, but the mechanism behind these effects remains unclear. The mouse skin epidermal JB6 C41 cell system is a well-developed model for studying TPA-induced tumor promotion under anchorage-independent growth conditions (32). Accumulating evidences showed that TPA-induced COX-2 expression and cell transformation through AP-1
activation in JB6 Cl41 cells. The present study demonstrated that p-HPEA-EDA, isolated from virgin olive oil, exhibited a strong inhibitory effect on TPA-induced neoplastic cell transformation. Also, p-HPEA-EDA downregulated AP-1 activity through inhibition of ERK1/2 phosphorylation induced by TPA in JB6 mouse epidermal cells. These results suggested that p-HPEA-EDA might play an important role in the cancer–preventive activity by targeting the AP-1-signaling pathway.

AMPK is a pivot point between cell survival and apoptosis (33), is a novel therapeutic target for cancer or metabolic disease (20,34), and is involved in cellular homeostasis (35,36). AMPK also acts as a positive regulator for p53 phosphorylation (Ser15) upon ultraviolet radiation and H2O2 exposure in human skin keratinocytes, which lead to cell apoptosis (37), suggesting that AMPK acts as an apoptotic molecule. Interestingly, Hwang et al. (29) reported that AMPK activation by 5-amino-4-imidazolecarboxamide riboside, a specific AMPK activator, downregulates COX-2 expression through inhibition of prostaglandin E(2) in colon cancer cells. Since COX-2 is highly expressed in many cancer cells including colon cancer cells, we studied the effect of p-HPEA-EDA on COX-2 expression and the underlying molecular mechanism in HT-29 colon cancer cells. The cell growth of HT-29 cells was significantly inhibited by p-HPEA-EDA, whereas it did not affect the cell growth of JB6 Cl41 cells. We hypothesized that HT-29 cells might highly express AMPK compared with JB6 Cl41 cells, and thereby p-HPEA-EDA may induce AMPK activation to downregulate COX-2 expression, resulted in inhibition of cell viability. Our results showed that AMPK is highly expressed in HT-29 cells compared with JB6 Cl41 cells and p-HPEA-EDA induced the phosphorylation of AMPK in a dose- and time-dependent manner and downregulated the expression of COX-2 in HT-29 cells, followed later with induction of apoptosis.

Ras/Raf/MEK/ERK signaling pathway, which is responsible for the upregulation of AP-1 activity, primarily regulates COX-2 expression (38). The induction of neoplastic cell transformation and COX-2 expression was abolished by the pharmacologic inhibition or dominant-negative knockout of MEK, suggesting that the MAP kinase pathway is partially responsible for cell transformation and COX-2 expression (39). Consistent with these observations, our results also showed that p-HPEA-EDA downregulated AP-1 activity through inhibition of ERK1/2 phosphorylation in JB6 Cl41 cells, and thereby inhibited neoplastic cell transformation induced by TPA. COX-2-knockout mice are resistant to experimental carcinogenesis, indicating that COX-2 is involved in carcinogenesis (40). Cell growth increases and apoptosis decreases in cells with spontaneous upregulation of COX-2 or in cells engineered to overexpress COX-2 (41,42). COX-2 induction occurs in early tumorigenesis, especially in colon cancer (43). Specific inhibitors of COX-2 such as celecoxib can inhibit cellular growth and proliferation through induction of cleaved caspase-3 in many cancer cells including HT-29 colon cancer cells (44–46). COX-2 is therefore a good molecular target for anti-inflammatory and chemopreventive agents. Our results also showed that p-HPEA-EDA downregulated the expression of COX-2 and induced cleaved caspase-3, cleaved PARP through activation of AMPK in HT-29 cells, and thereby increased the level of DNA fragmentation.

Fig. 6. In vitro and in vivo effects of p-HPEA-EDA on tumorigenicity of HT-29 cells. (A and B) HT-29 cells were treated or not treated with p-HPEA-EDA, as indicated concentration, in soft agar, and averaged colony numbers were measured. (C and D) 5 μg/ml p-HPEA-EDA were treated or not treated with Compound C or Z-DEVD-FMK into HT-29 cells in soft agar, and averaged colony numbers were measured. Columns, mean of triplicate samples; bars, standard deviation. *P < 0.05, compared with control cells. (E and F) HT-29 cells were seeded on the CAM of chicken embryos and treated with 50 μg/ml p-HPEA-EDA or dimethyl sulfoxide, respectively. Representative pictures of the CAM (E) and measured tumour area (F) are shown. Error bars indicate the means ± standard deviations of nine samples per group from two independent experiments. *P < 0.05, compared with control groups.
However, the knockdown or inhibition of AMPK in HT-29 cells attenuated the apoptosis induced by p-HPEA-EDA, suggesting that AMPK is an important molecular target of p-HPEA-EDA for down-regulation of COX-2 expression and induction of apoptosis. AMPK activation by 5-aminoo-4-imidazolocarbamdoxide riboside inhibits the proliferation of cancer cell lines in vitro and in vivo by increasing p21CIP, p27KIP and p53 activation (28). In addition, p53 upregulation or p53 phosphorylation (Ser15) by unique compounds, such as curcumin, meclizine or trans-farnesyl-thioisalicic acid, induces apoptosis and cell cycle arrest in HT-29 cells (47–49). Similarly, p-HPEA-EDA also increased p53 phosphorylation (Ser15) at the same dose and time at which it induced the phosphorylation of AMPK, indicating that p-HPEA-EDA-activated AMPK induces apoptosis through p53 activation.

Tumor cells can grow in soft agar, which is an indicator of their invasive potential (50). p-HPEA-EDA significantly inhibited colony formation of HT-29 cells in soft agar. Moreover, TPA-induced AP-1 activity and neoplastic cell transformation in JB6 CI4 cells were inhibited by p-HPEA-EDA, following inhibition of the TPA-induced mitogen-activated protein kinase signaling pathway. Therefore, these results supported our notion that the inhibition of AP-1 activity and COX-2 expression might be functionally linked to the antitumor promotion effects of p-HPEA-EDA.

Accumulating evidence suggests that high levels of COX-2 expression play a role in tumorigenesis, particularly in colon cancer. Therefore, these results prompted us to investigate the role of AMPK activation in pharmacological agents that could downregulate COX-2 expression and induce apoptosis in colon cancer cells. In this study, we determined the role and possible mechanisms of action of p-HPEA-EDA in regulating growth and apoptosis of cancer cell via AMPK activation and COX-2 expression in HT-29 cells. These studies provide insight into the biological actions of p-HPEA-EDA, a phenolic compound of virgin olive oil, and the molecular basis for the development of new chemotherapeutic agents.

Supplementary material
Supplementary Figures S1 and S2 can be found at http://carcin.oxfordjournals.org/

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17. P.Khanal et al. (2003) Olive-oil composition and the molecular basis for the study, we determined the role and possible mechanisms of action of a promising strategy for developing chemotherapeutic agents. In this study, we determined the role and possible mechanisms of action of p-HPEA-EDA in regulating growth and apoptosis of cancer cell via AMPK activation and COX-2 expression in HT-29 cells. These studies provide insight into the biological actions of p-HPEA-EDA, a phenolic compound of virgin olive oil, and the molecular basis for the development of new chemotherapeutic agents.

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