Y-27632 Increases Sensitivity of PANC-1 Cells to Epigallocatechin Gallate (EGCG) in Regulating Cell Proliferation and Migration

ACEF 1,2 Xing Liu
ABCEFG 1 Yongyi Bi

Corresponding Author: Yongyi Bi, e-mail: yongyibi68@126.com

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Background: The study aimed to investigate the inhibitory effect of (1R,4r)-4-((R)-1-aminoethyl)-N-(pyridin-4-yl)cyclohexanecarboxamide (Y-27632) and (–)-epigallocatechin-3-gallate (EGCG) on the proliferation and migration of PANC-1 cells. EGCG, found in green tea, has been previously shown to be one of the most abundant and powerful catechins in cancer prevention and treatment. Y-27632, a selective inhibitor of rho-associated protein kinase 1, is widely used in treating cardiovascular disease, inflammation, and cancer.

Material/Methods: PANC-1 cells, maintained in Dulbecco’s Modified Eagle’s Medium, were treated with dimethyl sulfoxide (control) as well as different concentrations (20, 40, 60, and 80 μg/mL) of EGCG for 48 h. In addition, PANC-1 cells were treated separately with 60 μg/mL EGCG, 20 μM Y-27632, and EGCG combined with Y-27632 (60 μg/mL EGCG + 20 μM Y-27632) for 48 h. The effect of EGCG and Y-27632 on the proliferation and migration of PANC-1 cells was evaluated using Cell Counting Kit-8 and transwell migration assays. The expression of peroxisome proliferator–activated receptor alpha (PPARα) and Caspase-3 mRNA was determined by Quantitative real-time polymerase chain reaction (RT-qPCR).

Results: EGCG (20–80 μg/mL) inhibited cell viability in a dose-dependent manner. Y-27632 enhanced the sensitivity of PANC-1 cells to EGCG (by increasing the expression of PPARα and Caspase-3 mRNA) and suppressed cell proliferation. PANC-1 cell migration was inhibited by treatment with a combination of EGCG and Y-27632.

Conclusions: Y-27632 increases the sensitivity of PANC-1 cells to EGCG in regulating cell proliferation and migration, which is likely to be related to the expression of PPARα mRNA and Caspase-3 mRNA.

MeSH Keywords: Cell Migration Assays • Cell Proliferation • Pancreatic Neoplasms

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Background

Pancreatic cancer, currently one of the most lethal human malignancies [1], is largely refractory to conventional therapies. Peroxisome proliferator-activated receptor alpha (PPARα), a member of the PPAR family [2], regulating tumorigenesis [3], is a ligand-activated transcription factor [4]. Caspase-3 is encoded by the CASP3 gene [5], as a potential therapeutic target for cancer patients [6] and plays key roles in the growth stimulation.

(–)-Epigallocatechin-3-gallate (EGCG) (C_{15}H_{24}O_{11}; Figure 1A), found in green tea [7], which is widely consumed in China [8], is one of the most abundant and powerful catechins [9] in cancer prevention and treatment. (1R,4R)-4-((R)-1-aminoethyl)-N-(pyridin-4-yl) cyclohexane carboxamide (Y-27632) (C_{6}H_{11}N_{2}O; Figure 1B), a selective inhibitor of rho-associated protein kinase 1 (ROCK1) [10], is widely used in treating cardiovascular disease [11], inflammation [12], and cancer [13]. Although Y-27632 [14–16] and EGCG [17–19] inhibit the growth of many cancer cells, whether the efficacy of Y-27632 increases the sensitivity of PANC-1 cells to EGCG is not yet clear. The present study hypothesized that the combination of Y-27632 and EGCG would reveal additive inhibitory effects in vitro.

In the present study, the capacity of Y-27632 to sensitize PANC-1 cells to the effects of EGCG in regulating cell proliferation and migration was investigated. Furthermore, the expression of PPARα mRNA and caspase-3 mRNA in EGCG and Y-27632 alone, and in EGCG combined with Y-27632 on PANC-1 cells, was examined.

Material and Methods

Cell culture

PANC-1 cells (SIBCB, Shanghai, China) were maintained in Dulbecco’s Modified Eagle’s Medium (DMEM) (Gibco BRL, MD, USA) (15) supplemented with 10% fetal bovine serum (Gibco BRL, MD, USA) and penicillin (100 U/mL)–streptomycin (100 mg/mL) (Gibco BRL, MD, USA) in a humidified atmosphere containing 5% CO₂ and 95% air at 37°C.

Cell proliferation assay

PANC-1 cells (1×10⁵/well) were seeded into 96-well plates (Corning, NY, USA). These cells were then treated with dimethyl sulfoxide (DMSO) (control) as well as different concentrations (20, 40, 60, and 80 μg/mL) of EGCG (NICPBP, Beijing, China) for 48 h. In addition, PANC-1 cells were treated separately with DMSO (control), 60 μg/mL EGCG, 20 μM Y-27632, and EGCG combined with Y-27632 (60 μg/mL EGCG + 20 μM Y-27632) for 48 h. Cell viability was assessed using the Cell Counting Kit-8 (CCK-8) [16] as described in a previous study. The absorbance (A) of each hole in the 96-well plate was determined at 475 nm using a microplate reader, using the formula:

Cell inhibition rate (%) = \frac{A_{\text{sample}} - A_{\text{blank}}}{A_{\text{control}} - A_{\text{blank}}} \times 100%

Hoechst 33258 staining

PANC-1 cells were treated separately with DMSO (control), 60 μg/mL EGCG, 20 μM Y-27632, and 60 μg/mL EGCG + 20 μM Y-27632 for 48 h. After washing with phosphate-buffered saline (Gibco BRL), the fixed PANC-1 cells were stained with 10 μg/mL Hoechst 33258 (Beyotime Institute of Biotechnology, Jiangsu, China) [17] for 10 min at room temperature.

Transwell migration assay

PANC-1 cells (1×10⁵/well) were placed in the upper chamber of a transwell filter. Drugs (DMSO, 60 μg/mL EGCG, 20 μM Y-27632, and 60 μg/mL EGCG + 20 μM Y-27632) were added separately into the upper chamber and PANC-1 cells were incubated for 10 h. After fixation and 0.1% crystal violet staining, PANC-1 cells were counted and the cell migration inhibition rate [18] of each group was calculated.

Quantitative real-time reverse transcription-polymerase chain reaction

PANC-1 cells were treated separately with DMSO (control), 60 μg/mL EGCG, 20 μM Y-27632, and 60 μg/mL EGCG + 20 μM Y-27632 for 48 h. Quantitative real-time polymerase chain reaction (RT-qPCR) [19] was used to observe the expression of PPARα mRNA and caspase-3 mRNA of these groups. The primer pairs [glyceraldehyde-3-phosphate dehydrogenase (GAPDH), PPARα, and caspase-3] were synthesized by Shanghai Sangon Biological Engineering Technology & Services Co., Ltd. (Shanghai, China). The primer pairs included: the forward: 5’-AAAGAGCGTGGCGCTATTGG-3’ and reverse: 5’-GCCGGGCCATCCACAGTCTC-3’ for GAPDH (258 bp); forward: 5’-TTGCTCAATCCATCGGCGAG-3’ and reverse: 5’-CCACAGGATAAGTCCACGAGG-3’ for PPARα (146 bp). Forward: 5’-TATAAGGAGCAATCTGGACT-3’ and reverse: 5’-CTGATCCAGACCGAGATGCTCA-3’ for caspase-3 (139 bp). GAPDH was used as an internal control to evaluate the relative expression of PPARα. RT-qPCR reagents were purchased from TIANGEN Biotech (Beijing) Co., Ltd. (Beijing, China). Relative mRNA was calculated using the formula: 2−ΔΔCT [20,21].

Statistical analysis

Data are shown as mean ± standard deviation. Statistical comparisons were performed using SPSS version 18.0 software (22). P<0.05 was considered to be significant [23,24].
Results

Y-27632 augments the antiproliferative effect of EGCG in PANC-1 cells

The effect of EGCG and Y-27632 on the PANC-1 cell proliferation was evaluated using the CCK-8 assay. Different concentrations of EGCG (20, 40, 60, and 80 μg/mL) inhibited cell viability in a dose-dependent manner (Figure 1C). EGCG (60 μg/mL) inhibited PANC-1 cell viability by 69% (Figure 1D). Y-27632 (20 μM) inhibited PANC-1 cell viability by 17% (Figure 1D), and 60 μg/mL EGCG + 20 μM Y-27632 inhibited PANC-1 cell viability by 82% (Figure 1D). These results suggest that 20 μM Y-27632 enhanced the sensitivity of PANC-1 cells to 60 μg/mL EGCG and suppressed cell proliferation.

Y-27632 improved the anti-migration and apoptosis effect of EGCG in PANC-1 cells

The effect of 60 μg/mL EGCG on the PANC-1 cell migration in the presence of 20 μM Y-27632 was evaluated using transwell migration assays (Figure 2A). Following treatment with 60 μg/mL EGCG + 20 μM Y-27632, the cell migration significantly decreased compared with that of the untreated PANC-1 control and the cells treated with 20 μM Y-27632 alone (Figure 2B). These data suggest that PANC-1 cell migration is inhibited by treatment with 60 μg/mL EGCG + 20 μM Y-27632. Under Hoechst 33258 staining (Figure 2C), the normal PANC-1 cells without 60 μg/mL EGCG and 20 μM Y-27632 intervention were dark blue (control), but early apoptotic nuclei due to chromosomal collapse were stained bright blue in the 60 μg/mL EGCG and 20 μM Y-27632 treatment groups (Figure 2C), and were easily distinguished from the normal cells. Also, the number of bright blue-stained nuclei

Figure 1. Effect of the combination of (–)-epigallocatechin-3-gallate (EGCG) and (1R,4r)-4-((R)-1-aminoethyl)-N-(pyridin-4-yl)cyclohexane carboxamide (Y-27632) on PANC-1 cell proliferation. (A, B) Chemical structure of EGCG (C_{22}H_{18}O_{11}) and Y-27632 (C_{14}H_{21}N_{3}O). (C) Different concentrations of EGCG (20, 40, 60, and 80 μg/mL) inhibited cell viability in a dose-dependent manner. The effect of EGCG and Y-27632 on the PANC-1 cell proliferation was evaluated by CCK-8 assay. (D) PANC-1 cells were treated separately with DMSO (control), 60 μg/mL EGCG, 20 μM Y-27632, and 60 μg/mL EGCG + 20 μM Y-27632 for 48 h. Data represent mean ± standard error of mean, n = 3. Compared with control, ** P<0.01. Compared with EGCG, * P<0.05.
increased (Figure 2C) in the 60 μg/mL EGCG + 20 μM Y-27632 treatment group.

**Combination of Y-27632 and EGCG increased the expression of PPARα mRNA and caspase-3 mRNA**

The expression of PPARα mRNA and caspase-3 mRNA was determined by RT-qPCR. The amplification and melting curves of PPARα and caspase-3 mRNA were observed in PANC-1 cells treated with 60 μg/mL EGCG or 20 μM Y-27632 alone, and 60 μg/mL EGCG + 20 μM Y-27632. Treatment with 20 μM Y-27632 + 60 μg/mL EGCG caused a sharp increase in the expression of PPARα mRNA and caspase-3 mRNA compared with the levels detected following treatment with 60 μg/mL EGCG or 20 μM Y-27632 alone (Figure 3C).

**Discussion**

Our study demonstrated that Y-27632 sensitized the PANC-1 cells to the inhibitory effects of EGCG on cell proliferation and migration. Furthermore, the combination of Y-27632 and EGCG promoted apoptosis of the PANC-1 cells. The results also indicate that the Y-27632-induced sensitization is related to the increased expression of PPARα mRNA and caspase-3 mRNA.

This study, using the CCK-8 assay, evaluated the probable effect of different concentrations of EGCG (20, 40, 60, and 80 μg/mL) on PANC-1 cells. The results are in agreement with a previous study [25], which showed that EGCG (20–80 μg/mL) inhibited the proliferation of PANC-1 cells in a dose-dependent manner. The results also showed that at 48 h, Y-27632 enhanced the sensitivity of PANC-1 cells to EGCG and suppressed the proliferation of PANC-1 cells.

In the present study, using transwell migration assays and Hoechst 33258 staining, the effect of 20 μM Y-27632 combined with 60 μg/mL EGCG on the PANC-1 cell migration and apoptosis was evaluated. The results also showed that 20 μM Y-27632 enhanced the anti-migration effect of 60 μg/mL EGCG on PANC-1 cells when treated for 48 h. Furthermore, the effects of 60 μg/mL EGCG in regulating apoptosis of PANC-1 cells enhanced when treated with 20 μM Y-27632 + 60 μg/mL EGCG.

Tumor growth and metastasis depend on angiogenesis [26,27], and gene expression profiling of PPARα has been used in several studies [28,29], but a very few studies included pancreatic cancer. When PANC-1 cells were exposed to EGCG [30], the
expression of PPARα, a direct negative regulator of heme oxygenase (HO-1) activation by EGCG [31], which confers cell susceptibility to EGCG, increased at the protein level in a dose-dependent manner. EGCG induces apoptosis and inhibits the growth of PANC-1 tumors [32] and activates caspase-3 is a dose-dependent manner. Therefore, the capacity of Y-27632 to sensitize PANC-1 cells to EGCG by activated PPARα mRNA and caspase-3 mRNA expression were investigated.

Figure 3. The combination of EGCG and Y-27632 increased the expression of PPARα mRNA and caspase-3 mRNA. PANC-1 cells were treated with DMSO (control), EGCG (60 μg/ml EGCG), or Y-27632 (20 μM Y-27632), and EGCG combined with Y-27632 (60 μg/ml EGCG + 20 μM Y-27632) for 48 h. The expression of PPARα mRNA and caspase-3 mRNA was analyzed by qRT-PCR. (A) The amplification curves of PPARα and caspase-3. (B) The melting curves of PPARα and caspase-3. (C) The relative gene expression of PPARα mRNA and caspase-3 mRNA in each group. Data represent mean ± SEM, n=3. Treatment with 20 μM Y-27632 + 60 μg/ml EGCG (compared with control, *P<0.05) caused a sharp increase in the expression of PPARα mRNA and caspase-3 mRNA compared with the levels detected following treatment with 60 μg/ml EGCG or 20 μM Y-27632 alone (compared with 20 μM Y-27632 + 60 μg/ml EGCG, *P<0.05).
Conclusions

The combination of EGCG and Y-27632 significantly increased the expression of PPARα mRNA and caspase-3 mRNA in PANC-1 cells. These data suggest that Y-27632 sensitizes PANC-1 cells to EGCG by increasing the expression of PPARα mRNA and caspase-3 mRNA. The synergistic effect of the combination of EGCG and Y-27632 provides new and useful information for its application in pancreatic cancer therapy.

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