Apoptotic Effect of Combination of Epalrestat and Palmitic Acid in Cholangiocarcinoma RBE Cells

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Abstract

Cholangiocarcinoma (CCA) classifies as a very deadly disease due to its detrimental effects, late diagnosis, and limited therapeutic options. AKR1B10, cloned from liver cancer, influences cell survival; the usage of epalrestat (EPS) to inhibit this gene results in the apoptosis of carcinoma cells, and utilizing palmitic acid (PA) partly reduces AKR1B10 silence-induced apoptosis. This investigation observed the combined effect of EPS and PA on the apoptosis of cholangiocarcinoma RBE cell. Our data showed that EPS treatment resulted in RBE cell growth inhibition by a concentration dependent and time independent manner. Strangely, the rescue effect of PA in EPS-induced RBE cell did not occur, and combining PA with EPS caused a synergistic effect in RBE cell apoptosis and growth inhibition in which hindering AKR1B10 activity, the loss of MMP, and the production of ROS might be involved; however, this experiment does not answer the questions of how EPS and PA affect AKR1B10 activity, MMP loss and ROS production, and the correlation between AKR1B10 inhibition and MMP loss and ROS production. This investigation concludes that administering EPS and an optimal concentration of PA in the blood by monitoring the amount of PA in the diet is a potential strategy to treat CCA patients.

Keywords: Cholangiocarcinoma; AKR1B10; Epalrestat; Palmitic acid; Apoptosis.

Introduction

Aldo-keto reductase family 1, member B10 (AKR1B10) was first identified in human hepatocellular carcinoma (HCC) [1]. Over expression of this gene occurs in various cancer cells [2], especially in liver cancer, but its expression is minimal in normal liver cells [3]. AKR1B10 expression is mostly observed in the small intestine and colon, but a low amount of its mRNA exists in the liver [1].

As analyzed by Northern blot hybridization, about 54% of HCCs contain high levels of AKR1B10 mRNA [1]. The expression of AKR1B10, detected by Immunohistochemical analysis, is highest in HCC, then in non-tumorous liver tissues, and lowest in normal liver tissues with 54.8%, 2.1%, and 0.3% positive expression, respectively [3].

AKR1B10 is crucial to the survival of cells, and silencing this gene with small interfering RNA (siRNA) can result in the apoptosis of carcinoma cells, such as human colon HCT-8 and lung NCI-H460 [4]. Previous investigations demonstrated that exposure to epalrestat (EPS), an AKR1B10 inhibitor, resulted in cell death [4].

Further studies have shown that AKR1B10 silence-mediated apoptosis may be related to the alternation of cellular lipids (especially phospholipids), reactive oxygen species (ROS), and mitochondrial damage [4], and that palmitic acid (PA), a substrate of sphingolipid signaling pathway, moderately reduced apoptosis caused by the silencing of AKR1B10 in HCT-8 cells [4].

Cholangiocarcinoma (CCA), a common cancer of the biliary tract, is divided into three subtypes that include intrahepatic, perihilar, and distal extrahepatic CCA [5]. A recent study showed that some new risk factors, such as obesity and diabetes with elevated PA, possibly led to the increase of CCA incidences over the last decade [5].

Because PA rescues EPS-induced cell death in HCT-8 human colon cells, we hypothesized that it would exhibit similar effects in CCA RBE cells; however, 200 µM EPS and 160 µM PA alone had no significant effect on RBE cell growth and combination of 200 µM EPS and 160 µM PA could induce apoptosis. Synergistic inhibition of AKR1B10, ROS production, and lose of mitochondrial membrane potential (MMP) might be involved in the apoptotic synergy induced by a combination of 200 µM EPS and 160 µM PA.

Keywords: Cholangiocarcinoma; AKR1B10; Epalrestat; Palmitic acid; Apoptosis.

Materials and Methods

Cells and materials

Cholangiocarcinoma RBE cells, obtained from the Institute of Biochemistry and Cell Biology (Chinese Academy of Sciences, Shanghai, China), were cultured in RPMI Medium 1640 basic (Gibco® by Life TechnologiesTM, Shanghai, China) supplemented with 10% heat-inactivated fetal bovine serum, 1.5 g/L NaHCO3, 2.5 g/L glucose, 0.11 g/L sodium pyruvate, 100 U/ml penicillin and 100 µg/ml streptomycin in a humidified atmosphere at 37°C and 5% CO2. Both epalrestat and palmitic acid were purchased from Sigma-Aldrich (St. Louis, MO, USA).

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3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was from Solarbio* Life Sciences (Beijing, China). Anti-human GAPDH monoclonal antibody (mouse) and HRP conjugated goat anti-mouse secondary antibody were from Beijing Zhongshan Golden Bridge Biotechnology Co. Ltd. (Beijing, China); Anti-AKR1B10 antibody was from Abcam (Cambridge, MA, USA).

L-glyceraldehyde, β-mercaptoethanol, phenylmethylsufonyl fluoride (PMSF), and sodium phosphate were bought from Sigma-Aldrich (St. Louis, MO, USA). NADPH was purchased from Solarbio* Life Sciences (Beijing, China), and leupeptin was from Amresco (Solon, OH, USA). Hoechst 33258 and JC-1 were purchased from Beyotime Institute of Biotechnology (Shanghai, China).

Western Blotting

RBE cells and liver cancer cells that were stored in our lab, such as HepG2, BEL-7402 or PLC were cultured. Their protein was isolated by lysing the cells with lysis buffer (1 mL RIPA+10 µL PMSF); and the cell membranes were broken by pipetting up and down. The samples were placed on ice for 5 minutes and then vortexed for 30 seconds; these two steps were repeated for a total time of 30 minutes.

After centrifuging at 12000 RPM at 4°C for 5 minutes, the protein in the supernatant was taken out of the centrifuge tube. Protein concentration was taken by adding 200 µL BCA to 25 µL of protein. Western blot was performed with the prepared protein. After running in 12% gel, the protein was transferred from the gel to PVDF membrane. 5% skim milk was used for blocking for about 1-1.5 hour in room temperature. 1st antibody was used to incubate 4°C overnight in a moist environment and 2nd antibody was incubated in 37°C for 1.5 hrs. Blots were visualized by ECL, detected on X-ray films.

MTT Assay

Cell growth was determined by using MTT as described in a previous study [6]. RBE cells were seeded in 96 well-plates with a density of 8000 cells/mL. After 12 hrs, cells were treated with different concentrations of EPS, PA, or a combination of EPS and PA for a variety of specific times like 6 hrs, 12 hrs, or 24 hrs. The cells were lysed with DMSO and placed in a spectrophotometer with wavelength set to 490 nm.

Hoechst Staining

Apoptosis was observed by performing Hoechst staining on RBE cells after they were seeded in 6-well plate and were treated with 200 µM EPS, 160 µM PA, and a combination of 200 µM EPS and 160 µM PA for 24 hrs. After the 24 hrs treatment, the media inside the wells were discarded, the wells were washed with 1 mL of 1xPBS, and then 0.5 mL fixing buffer was used to fix the cells for 10 minutes in room temperature.

After removing the fixing buffer, the wells were washed with 1mL of 1xPBS twice, three minutes each. In a lightless environment, cells were treated with 0.5 mL Hoechst 33258 for 5 minutes. After the allotted time, the wells were washed with 1mL 1xPBS twice, three minutes each. Pictures were then taken with a fluorescence microscope in a dark room.

AKR1B10 Activity Assay

Cell lysate was subjected to AKR1B10 enzyme activity assays using DL-glyceraldehyde as a substrate and the activity was expressed as oxidized NADPH (nmol)/protein (mg)/h as described previously [7].

Measurement of MMP

The loss of MMP was measured by using a cationic dye JC-1 whose fluorescence was monitored at the excitation wavelength of 488 nm and the emission wavelengths of 530 nm or 590 nm. After RBE cells were treated with 200 µM EPS, 160 µM PA, and a combination of 200 µM EPS and 160 µM PA for 24 hrs, cells were incubated with JC-1 (2.5 µg/mL) for 20 min at 37°C.

Finally, cells were observed under a fluorescence microscope, the red emission of the dye caused by the potential-dependent aggregation in the mitochondria represented MMP and green fluorescence corresponded to monomeric form of JC-1 which stranded in the cytosol after mitochondrial membrane depolarization. A reduction in red fluorescing mitochondria indicated a reduction in MMP.

Measurement of ROS Generation

A freely permeable tracer 2',7'-dichlorofluorescein-diacetate (DCFH-DA) can be de-acetylated by intracellular esterase to the non-fluorescent DCFH, which is then oxidized by ROS to the fluorescent compound, 2',7'-dichlorofluorescin (DCF), therefore, the fluorescence intensity of DCF is proportional to the amount of ROS production [8].

After exposing to 200 µM EPS, 160 µM PA, and a combination of 200 µM EPS and 160 µM PA for 24 hrs, cells were incubated with DCFH-DA at 37°C for 20 min in the dark. The fluorescence intensity was measured by using fluorescence microscopy (Nikon Te2000, Japan) with excitation wavelength of 488 nm and emission wavelength of 530 nm.

Results

EPS induced RBE cell growth inhibition by a concentration dependent and time independent manner

Because EPS induced cell death in HCT-8 human colon cells [4], the effects of EPS on RBE cells were observed. In order to determine an optimal EPS concentration and time period, RBE cells were seeded in a 96-well plate, and an MTT assay was performed. The cells were treated with 10-800 µM EPS for time periods of 6 hrs, 12 hrs, and 24 hrs. The results (Figure 1) showed that no growth inhibition could be seen in RBE cells treated with 10 µM, 20 µM, 50 µM, and 100 µM of EPS for 6 hrs, 200 µM of EPS also had no significant effect on RBE cell growth, however, 400 µM and 800 µM of EPS could inhibit RBE cell growth by nearly 60% and 80%, respectively. To our surprise, with treatment time extension to 12 hrs (Figure 1) or 24 hrs (Figure 1), no more growth inhibition could be observed in all concentrations of EPS treated- RBE cells, these data indicated that EPS induced RBE cell growth inhibition by a concentration dependent and time independent manner.

A synergic effect of cell growth inhibition was observed in the combination of EPS and PA

Based on the outcome in Figure 1, another MTT assay was performed and 200 µM EPS was used to observe the combined effects of EPS and PA. RBE cells were treated for 24 hrs with 200 µM EPS, 80
µM PA, 160 µM PA, 320 µM PA, 200 µM EPS+ 80 µM PA, 200 µM EPS+160 µM PA, or 200 µM EPS+320 µM PA. As shown in Figure 2, 200 µM EPS, 80 µM PA, or 160 µM PA alone did not have any significant effect on cells’ growth. However, a synergic response was observed when 200 µM EPS was combined with 160 µM PA (Figure 2). Surprisingly, 200 µM EPS+ 80 µM PA did not have any notable effect (Figure 2). As expected, 320 µM PA caused cell death; however, astonishingly, no synergic response occurred in cells that were treated with a combination of 200 µM EPS and 320 µM PA, the aftermath of cells treated with 200 µM EPS+320 µM PA was almost identical to cells treated with 320 µM PA only (Figure 2). As shown in Figure S1, 200 µM EPS, or 160 µM PA alone caused some growth inhibition in two human normal liver cell lines like L-02, QSG-7701, however, the synergic growth inhibition was not observed in these two liver cells when 200 µM EPS was combined with 160 µM PA.

A synergic effect of apoptosis can be observed with the combination of EPS and PA

RBE cells seeded in 6-well plate were used for Hoechst staining. The cells were treated with 200 µM EPS, 160 µM PA, and 200 µM EPS+160 µM PA for 24 hrs.

Figure 3 displayed the images taken from a fluorescence microscope, the navy blue ovals represented normal and healthy cells and the neon blue dots represented that apoptosis occurred. Cells treated with 200 µM EPS or 160 µM PA showed some, but not a significant amount of apoptosis (Figure 3); however, when 200 µM EPS and 160 µM PA were used together, a synergy of apoptosis can be observed (Figure 3).

Figure 1: RBE cells were seeded in a 96-well plate and were treated with different concentration of EPS (10-800 µM) for different time periods of 6 hrs (A), 12 hrs (B), or 24 hrs (C); then MTT assays were performed. Results were normalized to values from the vehicle-treated cells at the specified time point (#, P < 0.05 versus *).

Figure 2: RBE cells were treated with 200 µM EPS, 80 µM (A), 160 µM (B), 320 µM (C) of PA, or a combination of 200 µM EPS and aforementioned concentrations of PA for 24 hrs; then MTT assays were performed. Results were normalized to values from the vehicle-treated cells (*, P<0.05 versus **; #, P<0.05 versus +; #, P<0.05 versus ++; +, P>0.05 versus ++).

Figure 3: RBE cells were seeded in 6-well plate and were treated with different concentration of EPS (10-800 µM) for different time periods of 6 hrs (A), 12 hrs (B), or 24 hrs (C); then MTT assays were performed. Results were normalized to values from the vehicle-treated cells at the specified time point (#, P < 0.05 versus *).
Inhibition of AKR1B10 activity, loss of MMP and production of ROS might be involved in the synergy of apoptosis induced by a combination of EPS and PA

The data above showed that a combination of PA and EPS (an AKR1B10 inhibitor) caused a synergistic effect in RBE cell growth inhibition and apoptosis.

It is also reported that AKR1B10 silence-mediated apoptosis may be related to the alternation of ROS and mitochondrial damage [4]; therefore, we hypothesized that the mechanisms of above synergy might be related to inhibition of AKR1B10 activity, loss of MMP and production of ROS.

Consistent with previous findings that AKR1B10 is expressed in liver cancer, Western Blotting showed that liver cancer cells (HepG2, BEL-7402, and PLC) displayed a relatively high amount of AKR1B10 expression; however, the protein level of AKR1B10 was much higher in RBE cells than above liver cancer cells (Figure 4).

Discussion

CCA accounts for 10-20% of primary liver carcinoma and is the second most common primary hepatic tumor [9]. Consistent with previous findings [1-3], we found that AKR1B10 is over expressed in various liver cancer cells like HepG2, BEL-7402, and PLC; however, cholangiocarcinoma RBE cells displayed more AKR1B10 protein than any of the liver cancer cells listed above. The questions of whether or not it existed in cholangiocarcinoma tissue sample and the clinical significance of AKR1B10 over-expression in cholangiocarcinoma are not answered in this study.

Because of aggressiveness, late diagnosis and limited therapeutic options, CCA are very deadly [10]; therefore, looking for new therapeutic methods is emergent. AKR1B10 is involved in cell survival and inhibition of this gene by siRNA or EPS results in carcinoma cell apoptosis [4].

Our data showed that EPS treatment resulted in RBE cell growth inhibition by a concentration dependent and time independent manner.

Exposing the HCT-8 cells to 50 or 100 µM EPS resulted in elevation of cell death when compared with the vehicle control [4], but these concentrations of EPS had no effect on RBE cell growth, higher concentration of 400 or 800 µM of EPS could inhibit RBE cell growth, and the reason might be that RBE cell has more AKR1B10 expression.

It is well known that EPS, a carboxylic acid derivative, have beneficial effects in diabetic neuropathy; administration of EPS does not pose any safety concern [11], long-term treatment with EPS is well tolerated [12], so it is possible and reasonable that EPS could be used in cancer therapy.

It is reported in human colon HCT-8 and lung NCI-H460 cells that 80 µM of PA partly reduced apoptosis caused by the silence of AKR1B10 [4].

Surprisingly, the rescue effect of PA in EPS-induced RBE cell apoptosis did not occur (data not shown), and a synergic cell growth inhibition was observed when 200 µM of EPS was combined with 160 µM of PA.

Neither of these drugs had any significant effect on cell growth separately; the synergy in apoptosis was further confirmed by Hoechst staining. These data indicated that a combination of PA with EPS caused a synergistic effect in RBE cell apoptosis and growth inhibition.

The weird and interesting finding is that synergic apoptosis and growth inhibition response was only observed when 200 µM EPS combined with 160 µM PA, but not with 80 µM PA or 320 µM PA, which could mean that an optimal concentration of PA is also necessary to initiate the synergy of apoptosis and growth inhibition induced by EPS plus PA.

As we know, PA is the predominant saturated fatty acid in the diet in North America [13], so administering an optimal concentration of PA, along with EPS, in blood by through dietary means is a feasible potential method to treat CCA patients.

Because AKR1B10 activity is involved in cancer cell proliferation, inhibition of AKR1B10 activity induced cell apoptosis. Consistent with previous findings, exposure to 200 µM of EPS for 30 minutes, AKR1B10 activity in RBE cells was decreased compared to the vehicle treatment. To our surprise, 160 µM of PA also decreased AKR1B10 activity in RBE cells.
Figure 3: RBE cells were seeded in a 6-well plate and were treated with 200 µM EPS, 160 µM PA and a combination of 200 µM EPS and 160 µM PA for 24 hrs. Cells were stained with Hoechst and were photographed under a fluorescence microscope (A). The rates of cell apoptosis were calculated based on at least three randomly selected images (B) (*, P<0.05 versus **).

If 200 µM of EPS was combined with 160 µM of PA, more reduction in AKR1B10 activity was observed; this hinted that the synergy of apoptosis and growth inhibition in RBE cells mediated by PA and EPS might be caused by affecting the AKR1B10 activity.

As we know, many mechanisms are involved in PA-induced apoptosis; however, this is the first to report that the inhibition of AKR1B10 activity is one of them. It is reported that AKR1B10 silenced-mediated apoptosis may be related to the alternation of ROS and mitochondrial damage [4], two of which are also implicated in PA-induced apoptosis [14,15].
The synergistic effects in loss of MMP and production of ROS induced by a combination of 200 µM EPS and 160 µM PA is also confirmed in our study.

Therefore, combining PA with EPS caused a synergistic effect in RBE cell apoptosis and growth inhibition, in which inhibition of AKR1B10 activity, loss of MMP and production of ROS might be involved; however, how EPS and PA affect AKR1B10 activity, the relationship among AKR1B10 inhibition, MMP loss and ROS production are not to be answered in this study.

**Conclusion**

In conclusion, 200 µM of EPS combined with 160 µM of PA could induce RBE cell growth inhibition and apoptosis; however, neither has any significant effect alone.

The clinical significance is that administering EPS while keeping an optimal concentration of PA in the blood by through adjusting the amount of PA in the diet is a potential strategy to treat CCA patients.
Figure 5: RBE cells treated with 200 µM EPS, 160 µM PA or a combination of 200 µM EPS and 160 µM PA for 24 hrs were stained with JC-1 and then images (A) were acquired using a Nikon Te2000 microscope (magnification: 200×). (B) The fluorescence intensity was quantified and percent reduction in JC-1 red fluorescence relative to vehicle control cells was calculated. Data from at least 3 randomly selected images are expressed as mean ± SD (*, P<0.05 versus **; +, P<0.05 versus **).
Figure 6: ROS generation assay in RBE cells treated with 200 µM EPS, 160 µM PA or a combination of 200 µM EPS and 160 µM PA for 24 hrs. Images (A) were acquired using a Nikon Te2000 microscope (magnification 200×). The fluorescence intensity was quantified and percentage in ROS production of treated cells relative to that of vehicle control cells (B) was calculated. Data from at least 3 randomly selected images are expressed as mean ± SD (*, P<0.05 versus **).

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