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Original Article BIOPHYSICS

Effect of metformin on cell proliferation and apoptosis in steatosis HepG2 cell model

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ABSTRACT

OBJECTIVE: Metformin, which is commonly recommended drug for managing type II diabetes, has been reported to have anti-cancer properties and may improve the prognosis of some malignancies. Epidemiology studies have shown improved survival in cancer patients using metformin. However, the mechanism behind this phenomenon remains incompletely understood. In our study, Our objective was to investigate how metformin influences the proliferation and apoptosis of hepatocellular carcinoma cells induced with steatosis via palmitic acid and oleic acid.

METHODS: We established an in vitro cellular model of non-alcoholic fatty liver disease by inducing lipid accumulation in HepG2 cells through the use of oleic acid and palmitic acid. Oil Red O staining was conducted to observe the distribution of intracellular lipid droplets. Cell proliferation were detected using the BrdU cell proliferation detection kit. Protein expressions were detected by western blot method techniques.

RESULTS: We found that metformin reduced cell proliferation in palmitic acid and oleic acid-induced HepG2 cells compared to the control group. Moreover, our western blot data show that metformin treatment changes apoptosis.

CONCLUSION: Our results show that metformin inhibits cell viability of steatosis HepG2 cells. These findings may be preliminary for new studies in steatosis HepG2 cells and may provide new therapeutic targets or treatment strategies against hepatocellular carcinoma.

Keywords: Bax; Bcl-2; cancer; Metformin; oleic acid; palmitic acid.

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Hepatic carcinoma ranks sixth in terms of frequency and fourth in terms of fatality among malignant diseases globally and remains a significant threat to human health [1]. Hepatocellular carcinoma (HCC) constitutes approximately 90% of all liver malignancies. HCC is a predominant histological liver cancer. Important factors of the complex etiology of HCC also include heavy alcohol consumption and non-alcoholic fatty liver disease (NAFLD) [1]. Today, the treatment options used in the treatment of liver cancer are conventional surgical excision, radiofrequency ablation, transcatheter arterial chemoembolization and alternative modalities [2]. Studies on elucidating the molecular mechanism underlying HCC progression are important to identify effective therapeutic targets and indicators that predict relapse.

The liver, which is an important organ in terms of lipid biosynthesis and fatty acid oxidation, contributes to lipid metabolism with these features [3]. Hepatic steatosis is known to have a crucial role in the initiation and progression stages of NAFLD [4]. It is known that triglycerides accumulate in the cytoplasm of hepatocytes in hepatic steatosis, and studies have

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found that free fatty acids are found at high levels in the blood serum of patients with NAFLD. Two of these triglycerides, oleic acid (OA) and palmitic acid (PA), have been used to create in vitro cellular models of hepatic steatosis (a cell model of non-alcoholic fatty liver disease). These models are frequently used in studies of lipid uptake, triglyceride synthesis and fatty acid oxidation in hepatic steatosis [5–7].

Apoptosis activation involves the participation of pro-apoptotic factors like Bax and anti-apoptotic factors such as Bcl-2 [8]. Bcl-2 can bind to Bax and inhibit apoptosis. When Bax induces the release of cytochrome c, caspase-3 is activated and facilitates the process of apoptosis [9]. Upregulation of Bax with simultaneous downregulation of Bcl-2 leading to an increase in the Bax/Bcl-2 ratio is an extensively studied parameter in the apoptotic death of cancer cells [10]. Apoptotic signalling pathways are obvious drug targets for targeted different cancer therapies.

Metformin (1,1-dimethylbiguanide hydrochloride) is now mostly used in the treatment of diabetes and is prescribed for type 2 diabetic patients [11]. Metformin, which research has shown to have anti-cancer properties, is an antidiabetic biguanide agent derived from Galega officinalis [12, 13]. Metformin exerts a suppressive impact on cellular proliferation, angiogenesis, epithelial-mesenchymal transition and tumor growth in various cancers [14]. With these properties, metformin can be thought to improve the prognosis of cancer patients and prevent tumor formation.

There are studies showing that metformin suppresses cell growth and triggers programmed cell death in cancer cells through elevated AMP/ATP ratio and activation of AMP-activated protein kinase (AMPK) [15]. Metformin functions as an agonist of AMPK, a serine/threonine kinase [16]. Metformin has been discovered to inhibit complex 1in the mitochondrial electron transport chain [16], which serves as a significant generator of ROS (reactive oxygen species) [17]. Metformin inhibits the respiratory complex I of the electron transport chain (ETC) in the mitochondria and causes a slight electron transport leak, leading to ROS production and a slight decrease in ATP production. Decrease in ATP causes activation of AMPK, which inhibits the mammalian target of rapamycin (mTOR) pathway and leads to a decrease in cell proliferation. It is known that excessive inhibition of the mTOR pathway can induce apoptosis and cell cycle arrest in the cell [18, 19].

Highlight key points

- Metformin reduces the proliferation of steatosis HepG2 cells.
- Metformin has a stimulatory effect on the apoptosis pathway in steatosis HepG2 cells.
- Metformin treatments in steatosis HepG2 cells may contribute to preventing NAFLD progression to HCC.

The model we created using oleic acid and palmitic acid is an NAFLD steatosis model. HCC and NAFLD are known to be interrelated. Obesity, NAFLD increases the risk of HCC [20]. Studies have shown that NAFLD may act synergistically with other HCC risk factors to accelerate cancer development [21]. NAFLD is recognized as an inducible cause of HCC [22], and nonalcoholic steatohepatitis, a manifestation of NAFLD, can progress to HCC [23]. To study hepatic steatosis in vitro, a cellular hepatic model was established by treating human HepG2 cells with free fatty acids [24].

In this study, we aimed to investigate the effect of metformin on the proliferation of steatosis HepG2 cells, and to identify the possible apoptosis mechanism of the effect of metformin on steatosis HepG2 cells, we measured the expression of cytochrome C, Bax, Bcl-2 and caspase 3 proteins in steatosis HepG2 cells. This research was purposed to explore the impact of metformin on cellular proliferation and apoptosis in steatosis-induced hepatocellular carcinoma cells.

MATERIALS AND METHODS

Agents, Chemicals and Assay Kits

Oleic acid, palmitic acid and actin antibodies were supplied from Santa Cruz Biotechnology (CA, USA). Metformin was supplied from Cayman Chemicals (Ann Arbor, MI, USA). Oil Red-O was supplied from Sigma-Aldrich Biotechnology (Steinheim, Germany). BrdU Cell Proliferation ELISA Assay kit and protease cocktail inhibitor were obtained from Roche Molecular Biochemicals (Mannheim, Germany). Lowry Smart BCA Protein kit was obtained from Intron Biotechnology (Seoul, Korea). RPMI-1640 was obtained from Pan Biotechnology (Aidenbach, Germany). Fetal Bovine Serum (FBS) was supplied from Gibco (Grand Island, NY, USA).

Cell Lines and Cell Culture Conditions

HepG2 cells were supplied from American Type Culture Collection (Manassas, VA, USA). HepG2 cells were

maintained in RPMI-1640 culture medium (Pan Biotech.). Each medium was supplemented with 10% FBS (Gibco) and contained 100 units/ml penicillin and 100 µg/ml streptomycin. All cell cultures were maintained at a temperature of 37°C under a 5% CO_2 atmosphere.

Stock solutions of palmitic acid (66 mM) and oleic acid (132 mM) were prepared in DMSO. The final concentration of metformin stock solution in PBS is 1 mg/ml (pH 7.2). All solutions underwent sterile filtration using a 0.22 µm pore membrane filter and were subsequently preserved at -20°C.

PA and OA-induced Steatosis Model

Cells were plated in six-well plates at a density of 1×10^6 cells per well, followed by incubation of HepG2 cells in RPMI-1640 medium with palmitic acid at a concentration of 0.33 mM and oleic acid at a concentration of 0.66 mM for 24 h [25].

Oil Red O Staining

The cells were washed with phosphate-buffered saline (PBS), followed by fixation with 4% paraformaldehyde for 30 minutes and subsequent staining using a freshly prepared working solution of Oil Red O for 30 minutes. Afterward, the cells underwent washing with deionized water. Stained Oil Red O was further dissolved using 100% isopropanol at room temperature for 15 minutes. The absorbance of the sample was measured using a spectrophotometer (Synergy H1, BioTek, Winooski, VT) at 510 nm.

Cell Proliferation Assay

Cells were plated onto a 96-well plate at a density of $1x10^4$ cells per well and allowed to grow overnight. The cells were then incubated with metformin (2 mM) [26] for 24 hours. Cell proliferation was measured with a quantitative colorimetric BrdU (Roche Biochemicals) assay kit. It was determined by measuring optical density at 370 nm with a microplate scanning spectrophotometer (Synergy H1, BioTek, Winooski, VT).

Preparation of Cell Lysates and Western Blot Analysis

Cells were washed with cold PBS and centrifuged at 300x g for 15 min at 4 °C. The supernatant was collected and homogenized in lysis buffer included protease inhibitor cocktail. Protein concentration was determined by Lowry's Assay (Smart BCA protein kit- iNtRON) used to normalized protein level [27]. The protein sample (50 µg) was prepared and subjected to boiling at 95°C for 3 min. The proteins were loaded on SDS-PAGE. Subsequently, the gels were transferred to a nitrocellulose membrane (Schleicher & Schuell, Keene, NH). The membrane was blocked in 10% BSA in TBST buffer for 1 h at room temperature. After blocking, membranes were incubated overnight at 4°C with primary antibodies; caspase 3 (Invitrogen, Carlsbad, CA, USA), cytochrome c (Invitrogen, Carlsbad, CA, USA), Bcl-2 (Invitrogen, Carlsbad, CA, USA), Bax (Invitrogen, Carlsbad, CA, USA). Actin (Santa Cruz Tech., CA, USA) antibody was used for control. The membrane underwent three washes with TBST buffer before being exposed to the secondary antibody for 1 hour. The membranes were washed and subjected to incubation with secondary antibodies (Thermo Fisher Sci., Waltham, USA) for 1 hour at room temperature. The specific bands labeled by the antibody were visualized using NBT/BCIP or chemiluminescent substrate containing ECL substrate (Pierce ECL Western Blotting Substrate luminol-based activator- Thermofisher Scientific, Waltham, USA). The relative expression levels of proteins were determined by analyzing band intensities through computerized densitometry, employing the freely available version of ImageJ software (NIH, USA). The molecular weights of cytochrome c, caspase 3, Bcl-2, Bax and actin are 15 kDa, 32 kDa, 23 kDa, 21 kDA and 43 kDa, respectively.

Statistical Analysis

The experimental data were demonstrated as means±-standard error of the mean (SEM). All data reflect a minimum of three experiments performed in triplicate. The analysis of all data was conducted using Prism software (ver. 8; GraphPad Software Inc., San Diego, CA, USA). The Mann Whitney U test was employed to assess statistical differences between means. A significance level of $p<0.05$ was considered statistically significant.

RESULTS

Creating a Steatosis Model in HepG2 Cells Using Palmitic Acid and Oleic Acid

Steatosis was determined by Oil Red-O staining in cells incubated with palmitic acid at a concentration of 0.33 mM and oleic acid at a concentration of 0.66 mM for 24 hours. The findings indicate notable accumulation of lipid droplets within the cytoplasm of HepG2 cells in comparison to the control group ($p=0.0159$; Fig. 1).

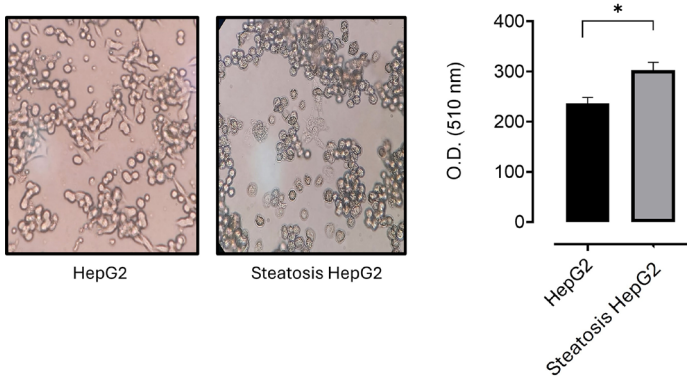

FIGURE 1. HepG2 and steatosis HepG2 cells Oil Red O staining (magnification x400), Comparison of the degree of steatosis with Oil red O staining in the steatosis model created in HepG2 cells incubated for 24 hours using palmitic acid (0.33 mM) and oleic acid (0.66 mM). Results are given as ±SEM. Experiments were performed in triplicate (*p<0.05).

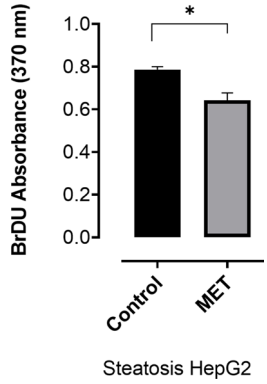

FIGURE 2. Effect of metformin on cell proliferation in steatosis HepG2 cells. Cells were treated with metformin (2mM) for 24 hours and cell counting was performed using the BrDU kit. Results are given as ±SEM. Experiments were performed in triplicate. (*p<0.05) Control: No drug; MET: Metformin.

In this study, we developed a steatosis cell model with HepG2 cells using OA and PA. We were able to quickly and accurately measure the degree of steatosis caused by PA and OA. The oil red-O-based colorimetric quantitative assay used in this study is not only convenient and measurable but also sensitive and reproducible.

Effect of Metformin on Cell Proliferation In Steatosis Hepg2 Cells

It is known that metformin can protect hepatocytes from death caused by saturated fatty acids. In our study, steatosis HepG2 cells were treated with metformin (2 mM) for 24 hours and its effects on cell proliferation was investigated. Metformin inhibited cell proliferation in steatosis HepG2 cells compared to the control group (p=0.0286; Fig. 2).

Effect of Metformin on Apoptosis in Steatosis HepG2 Cells

The effect of metformin on apoptosis in HepG2 cells with steatosis was assessed through western blot analysis of cytochrome C, caspase 3, Bcl-2, and Bax protein expression levels.

In steatosis HepG2 cells, cytochrome c expression and Bax/Bcl-2 expression levels rate increased in the metformin group compared to the control group (p=0.0286; Fig. 3). The change in caspase 3 expression level compared to the control was not found to be statistically significant (p=0.0571).

DISCUSSION

Metformin, an oral biguanide drug that has been prescribed for almost 60 years, has some features that stand out for its use in the prevention and treatment of cancer. In this case, it becomes important to determine whether metformin can directly affect the metabolism in tumor cells. The most important reason why the liver is one of the targets of the antidiabetic effect of metformin is that it plays a role in the management of glucose metabolism [28]. In vitro studies have shown that metformin used at concentrations greater than 1 mM causes mitochondrial complex 1 inhibition, decreases in aerobic metabolism, decreases in energy production and therefore eventually causes cell death [29]. Indirect effects of metformin in cancer: reducing glucose level, reducing hyperinsulinemia, reducing IGF-1 level, reducing NF-κB, reducing pro-inflammatory cytokines and increasing immune response in cancer cells. AMP-activated protein kinase (AMPK) dependent effects of metformin: It reduces mTOR, reduces myelocytomatosis oncogene (c-MYC) and increases p53 phosphorylation. AMPK-independent effects of metformin: It reduces ROS, increases mammalian target of rapamycin complex 1 (mTORC1), decreases cyclin D1, decreases autophagy and increases cancer cell apoptosis [30].

Current studies show that cytochrome c is first released from the intramitochondrial membrane following depolarization of the mitochondrial membrane, which then activates cytosolic caspases [31, 32]. The ratio of

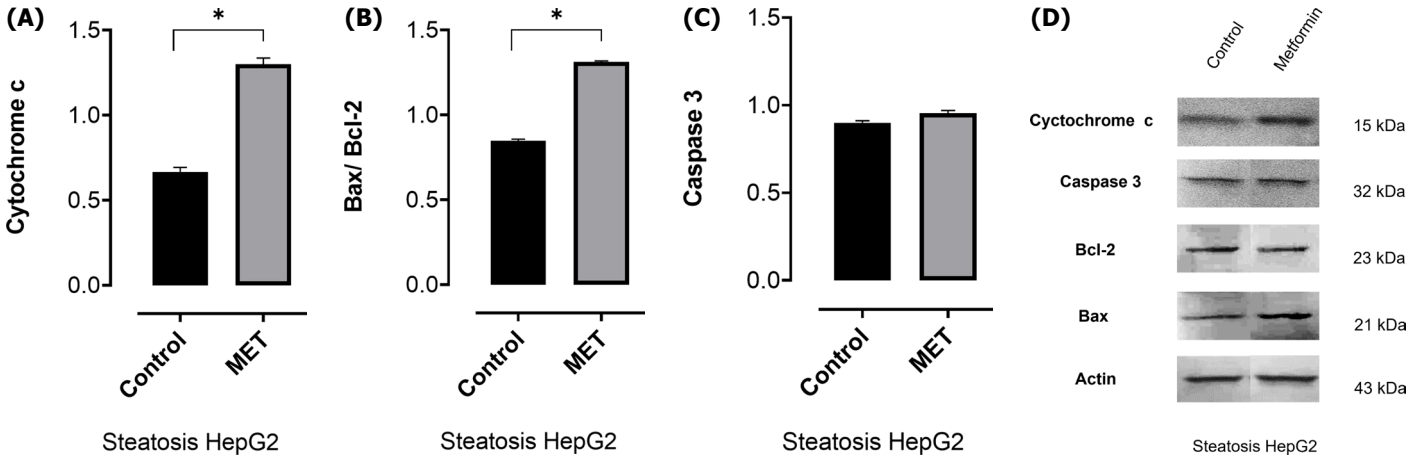

FIGURE 3. Changes in signaling proteins during apoptosis. Western blot analysis of apoptotic proteins in steatosis HepG2 cells was performed to elucidate the underlying mechanism(s) of apoptosis. Steatosis HepG2 cells were cultured for 24 hour. Protein extracts were loaded from cultured steatosis HepG2 cells. Samples were electrophoresed on an SDS-PAGE gel before being blotted onto a nitrocellulose membrane. Specific antibodies were used to probe the presence of **(A)** cytochrome c, (*p<0.05) **(B)** Bax/ Bcl-2 (*p<0.05) and **(C)** caspase-3 (p<0.05) on the blots. **(D)** The representative membrane images of Cytochrom c, Bax, Bcl-2, caspase 3. The positions relative to the molecular weight standards are represented on the right. Results are given as ±SEM. Experiments were performed in triplicate. Control: No drug; MET: Metformin.

Bax/ Bcl-2 proteins is crucial in dictating cellular fate, either survival or death [33].

Metformin has been demonstrated to decrease hepatic triacylglycerol and free fatty acid concentrations, while enhancing lipoprotein lipase activity in fructose-fed rats exhibiting hyperinsulinemia and hyperglycemia [34]. Zang et al. [35] found that metformin stimulated AMPK alpha phosphorylation in HepG2 cells and reduced intracellular lipid content in a dose (0.5–2 mM) and time-dependent (0–24 hours) manner. In that investigation aimed at assessing the impact of metformin-induced AMPK activation on the modulation of hepatocellular lipids, it was demonstrated that metformin prevented lipid accumulation induced by elevated glucose levels through an AMPK-dependent mechanism. Their research provided compelling biochemical proof that metformin's influence on the lipid composition of HepG2 cells dependent on AMPK activation. In our study, metformin was used to examine its anti-cancer effects on fatty liver cancer cells by creating a steatosis model, taking into account its effect on glucose metabolism in the liver. There are studies in the literature investigating the anti-cancer effects of metformin. The difference of our study is to investigate these effects in fatty liver. In our study, free fatty acids PA and OA were used to create an in vitro model of NAFLD. Our Oil red-O staining results confirmed that the model is functional.

Fendt et al. [36] found that metformin decreased proliferation in prostate carcinoma (LNCaP and DU145) and prostate adenocarcinoma (PC3) cells in a dose-dependent manner in the concentration range of 0.5–2.5 mmol/L. Li et al. [37] found that metformin reduced cell proliferation in osteosarcoma cells both dose-dependently (5–40 mM) and time-dependently (24–72 hours). Additionally, they showed in their study that metformin significantly reduced colony formation. Their results suggest that metformin inhibits cell viability of osteosarcoma cells. They suggested that metformin causes cell cycle arrest in the G2/M phase by upregulating cell cycle-related proteins. Mogavero et al. [38] observed that treatment with metformin at a concentration of 5 mM resulted in diminished proliferation, migration, and invasion of colorectal cancer cell lines. They noted an elevation in the proportion of cells in the G0/G1 phase and a reduction in the expression of various cell cycle regulatory proteins in CRC cells following metformin treatment. Their findings suggested an augmentation in ROS production in cells, proposing that metformin's mechanism of action may involve elevating ROS levels and inhibiting mTOR in these cell lines. Similar findings are found in ovarian and luminal breast cancer. They showed that metformin reversibly inhibited colony formation. They predicted that the anti-proliferative effect of

metformin is caused by the suppression of the mTOR pathway, which plays a critical role in cell growth. As a main result of our study, metformin (2mM) was able to the decreases of cell proliferation in PA and OA-induced HepG2 cells compared to the control group in the steatosis HepG2 cell. We previously showed that colony formation was reduced in steatosis HepG2 cells compared to control HepG2 cells.

Li et al. [37] showed that the percentage of cell apoptosis increased significantly after treatment with metformin. From all their results, they suggested that apoptosis may occur by activating extrinsic and intrinsic pathways after metformin treatment. Shen et al. [39] found that metformin (10–20 μM) inhibited the migration and colony formation of HCC cells (HepG2 and Huh7) and induced apoptosis. They found that metformin inhibited HCC cell growth in vivo and had anti-tumor and pyroptosis-inducing effects on HCC cells. Wu et al. [40] observed a notable increase in apoptosis rates in HepG2 cells treated with oleic acid compared to the control group, which significantly decreased following treatment with metformin. They proposed that metformin effectively ameliorated steatosis and could enhance HepG2 functionality in a cellular model of NAFLD. Based on these findings, they suggested that metformin's mechanism of action might involve mitigating oxidative stress damage, modulating the expression of proteins associated with the mitochondrial apoptosis pathway, and suppressing cellular apoptosis. Our western blot results show that metformin treatment induces apoptosis. Metformin increase in Bax/Bcl-2 protein expression ratio and also cytochrome c protein expression in steatosis HepG2 cells. Our results show that metformin inhibits cell viability of steatosis HepG2 cells.

Conclusion

Based on all of our results, we suggest that the mechanism by which metformin operates might involve the inhibition of cell proliferation and the modulation of protein expression associated with apoptosis pathways. In summary, in this study, upregulation of cyt c and bax play an important role in the mechanism of metformin-induced apoptosis in steatosis HepG2 cells. However, the effect of metformin on caspase 3 needs to be further clarified. These findings may provide insight into finding new therapeutic targets or treatment strategies against HCC.

Ethics Committee Approval: Ethics committee approval is not required for this study.

Conflict of Interest: No conflict of interest was declared by the authors.

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Authorship Contributions: Concept – HC, AMTB; Design – HC, AYTB; Supervision – HC; Analysis and/or interpretation – DM, SPK; Literature review – HC, AMTB, BA; Writing – AMTB, HC; Critical review – HC, AMTB, BA.

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