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Metformin Suppresses Epithelial Cell Adhesion Through ROS Production Involving Apoptosis in Cervical Cancer Cells

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ABSTRACT

Cervical cancer is one of the most prevalent cancer among female after breast cancer and its mortality rate is the highest in women malignant tumor of reproductive system. Epidemiological study indicates that metformin, an anti-diabetes drug, has an anti-proliferation effect with apoptotic-response mitochondrial signaling cascade in various cancers. However, the effect of metformin-mediated apoptosis involves in epithelial cell adhesion is not well characterized. Therefore, the mechanism underlying metformin-induced apoptosis related to epithelial cell adhesion expression in HeLa cells was investigated. The anti-proliferation effect of metformin in the HeLa cells was determined by MTT assay. Cell apoptosis was detected using Flow cytometry assay. The levels of E-cadherin (epithelial cell adhesion marker) expression were performed using Western blot analysis as well as the intracellular ROS levels were measured by DCFH-DA fluorimetric assay. The results revealed that metformin inhibited cell proliferation and induced cell apoptosis. Moreover, metformin suppressed the expression of E-cadherin through the increased ROS generation that was completely rescued by N-acetyl cysteine (NAC), ROS scavenger. Taken together, our results suggested that metformin-mediated apoptosis involved in the elevated ROS suppressing the expression of epithelial cell adhesion.

Keywords: Metformin, ROS, Cell adhesion, E-cadherin, Apoptosis

INTRODUCTION

Cervical cancer is the second most frequently diagnosed malignant cancer that occur among women worldwide (Canavan and Doshi, 2000). Recently, although advances in surgery, chemotherapy and radiotherapy have been predominant therapeutic treatment for cervical cancer (Rischin et al., 2010), the cost of therapeutic treatment is also extremely expensive. Therefore, providing a cheaper and more effective medicine would be alternative cervical cancer treatment.

Metformin has been extensively prescribed an anti-diabetes drug (Sreenivasan et al., 2014). Accumulating of evidences indicated that metformin exhibited a potential role in an anti-tumor effect with growth inhibition, cell cycle arrest, apoptosis and adenosine monophosphate-activated protein kinase (AMPK) signaling in various cancer cells (Fang et al., 2014; Pollak, 2010). Even though the effect of metformin on HeLa cell has been reported (Tang et al., 2018), the apoptosis mediated molecular mechanism of metformin remains unclear.

Reactive oxygen species (ROS), cellular metabolites, are considered essential for regulation in cancer development by increased metabolism sustaining, which promotes cancer proliferation (Yang et al., 2018). Excess cellular levels of ROS cause proteins, nucleic acids, cell membrane and organelles damage and mediate apoptotic signaling cascade (Redza-Dutordoir, 2016).

It is well known that cell adhesion is a pivotal role in several fates of biological processes, including cell proliferation, cell migration, and cell survival (Khalili and Ahmad, 2015). Although a growing body of evidence has been focused on integrin, a transmembrane protein facilitating cell adhesion, cellular and extracellular matrix interaction, E-cadherin, a classical cadherin transmembrane protein, has generally been recognized to mediate strong cell-cell adhesion (Noren, 2001). Loss of E-cadherin-catenin complex stimulated apoptotic response (Galaz, 2005). However, the molecular mechanism linking apoptosis and cell adhesion is still poorly understood.

In the present study, the mechanism underlying of metformin-induced apoptosis involving cell adhesion in cervical cancer HeLa cells was assessed. It revealed that metformin-induced cell apoptosis involved in ROS

suppressing E-cadherin expression. Ourfindings provided further evidence for the potential effect of metformin in cervical cancer treatment.

MATERIALS AND METHODS

Chemicals

Metformin was purchased from Apex Biotech (Apex Biotechnology Corp., Hsinchu, Taiwan). NAC was obtained from Sigma-Aldrich (St Louis, MO, USA).

Cell culture

Human cervical cancer HeLa cells were obtained from ATTC (Bethesda, MD, USA). The cells were grown in Eagle's Minimum Essential Medium (EMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 1% L-Glutamine and 100 U/ml penicillin/streptomycin (all from Gibco, Grand Island, NY, USA) at 37 °C in a 5% CO_2 incubator.

Cell viability

Cell viability was examined using MTT assay. Cells were seeded in a 96-well plate at a density of 1×10^4 cells/well and incubated for 24 h. Cells were then treated with metformin at the concentrations of 1 and 5 mM for 24 h. After incubation, cells were added with 5 mg/ml MTT (Sigma Chemical CO, St Louis, MO, USA) and incubated for 4 h. Thereafter, 100 µl DMSO was added to dissolve the formazan precipitates. The absorbance of formazan salts was detected at 570 nm.

Flow cytometry analysis

Apoptotic cells were detected by flow cytometry analysis. Cells were plated in a 6-well plate at a density of 1×10^6 cells/well and incubated for 24 h. After that, cells were treated with metformin at the concentrations of 1 and 5 mM for 24 h. After the treatment, cells were trypsinized and washed with PBS. Cells were then stained with Annexin V-FITC/7-AAD and immediately analyzed by flow cytometer (Millipore, Massachusetts, USA).

Intracellular ROS measurement

Intracellular ROS was measured using the 2',7'-dichlorofluorescein-diacetate (DCFH-DA) fluorescence dye. Cells were seeded in a 96-well plate at a density of 1×10^5 cells/well and incubated for 24 h. After incubation, the

cells were treated with 5 mM metformin for 1, 3 and 6 h. Next, cells were incubated with 50 µM DCFH-DA for 1 h at 37 °C. Subsequently, cells were washed with 200 µL PBS. ROS generation was monitored by measuring the fluorescence densities at 485 nm and 530 nm with a microplate reader (Bio-Tek Instruments Inc., Winooski, VT, USA).

Western blot analysis

After treatment, total cell extracts were prepared by treating cells with RIPA lysis buffer (ThermoFisher, Rockford, USA) at 4 °C for 15 min. Protein concentrations were determined using Bradford assay (Hercules, CA, USA). Whole cell lysates (30 µg) were loaded onto 10% polyacrylamide gels and transferred to PVDF membranes (Millipore, Temecula, CA, USA). The membrane was blocked with 5% skim milk for 2 h. The membrane was incubated with specific primary antibodies, anti-E-cadherin (1:1000) (Merck KGaA, Darmstadt, Germany) and anti-β-actin (1:1000) (Invitrogen, Rockford, USA). The primary antibodies were then detected using HRP-conjugated secondary antibody (1:5,000) (Abcam, HK, USA). The immunoblot was detected by using an enhanced chemiluminescence reagent (Thermo Fisher Scientific Inc., Rochford, IL, USA) and analyzed using Image J software (ImageJ, NIH, Bethesda, MD, USA).

Statistical Analysis

Data were presented as the mean ± standard deviation of three independent experiments. Statistical analysis was performed using pair-independent t-test to assess significance compared to untreated control group. *P* values lower than 0.05 were considered statistically significant.

RESULTS

Ant-proliferation effect of metformin on cervical cancer HeLa cells

In order to examine the effect of metformin on cell proliferation in HeLa cells, the cell proliferation was investigated using MTT assay. HeLa cells were treated with metformin at the concentrations of 1 and 5 mM for 24 h. Metformin treatment dramatically decreased cells proliferation in a dose-dependent manner at 24 h compared to control untreated group (Figure 1). The results indicated that metformin has a potential to be an anti-proliferation agent against HeLa cells.

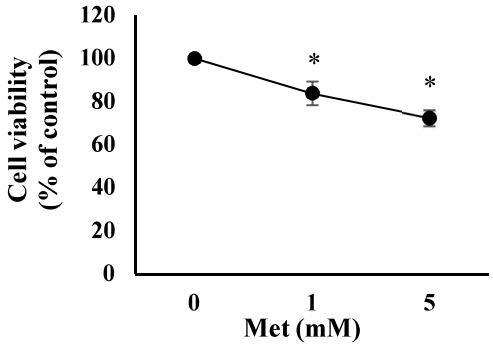


Figure 1. Effect of metformin on cell proliferation. HeLa cells were treated with metformin at 1 and 5 mM for 24 h. Cell viability was assessed by MTT assay. The percentages of cell viability were expressed as the mean ± standard deviation of at least three experiments. **P*<0.05 compared to untreated control group. Met: Metformin

Metformin induced apoptosis in HeLa cells

To investigate whether metformin inhibited the cell proliferation through induction of apoptosis by flow cytometry, cells were treated with metformin at the concentration of 1 and 5 mM for 24 h. At the end of the treatment, HeLa cells were stained with Annexin V/7-AAD and analyzed with flow cytometry. Metformin treatment significantly increased apoptotic cells in a dose-dependent manner compared to untreated control group (Figure 2). The result suggested that metformin induced apoptotic cell death.

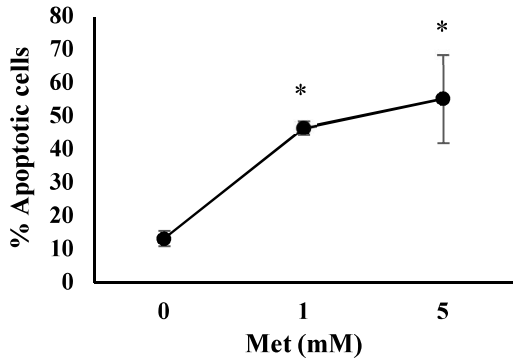


Figure 2. Effect of metformin on apoptosis induction. Cells were treated with metformin at 1 and 5 mM for 24 h. Apoptotic cells was determined by flow cytometry using Annexin V/ 7- AAD staining. The percentages of apoptotic cells were expressed as the mean ± standard deviation of at least three experiments. * $P<0.05$ compared to untreated control group. Met: Metformin

Metformin inhibited cell adhesion in HeLa cells

E-cadherin is one of the key classical cadherin transmembrane proteins that mediated cell- cell adhesion (Gumbiner, 2005) . It has been reported that E- cadherin- deficiency induced apoptosis (Galaz, 2005) . To determine whether E-cadherin are susceptible to metformin-induced apoptosis, the effect of metformin on E- cadherin expression was examined using Western blot analysis (Figure 3A) . The expressions of E- cadherin were significantly decreased in metformin treatment at 1 and 5 mM compared to untreated control group. However, metformin at 1 mM was not significantly decreased the expression of E- cadherin (Figure 3B) . The results demonstrated that metformin at high dose could suppress cell adhesion- response apoptosis. Based on the results, 5 mM metformin was utilized in next experiments.

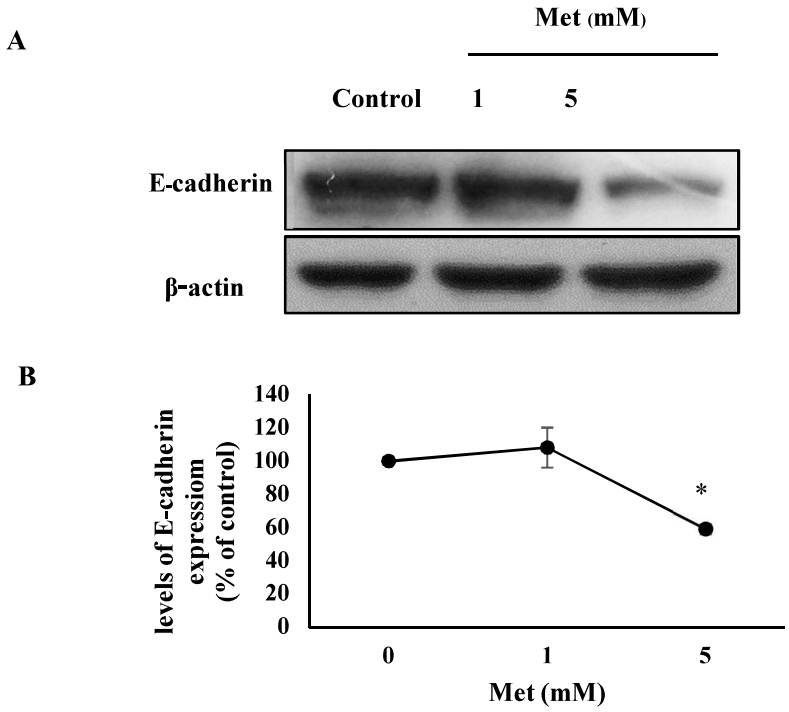
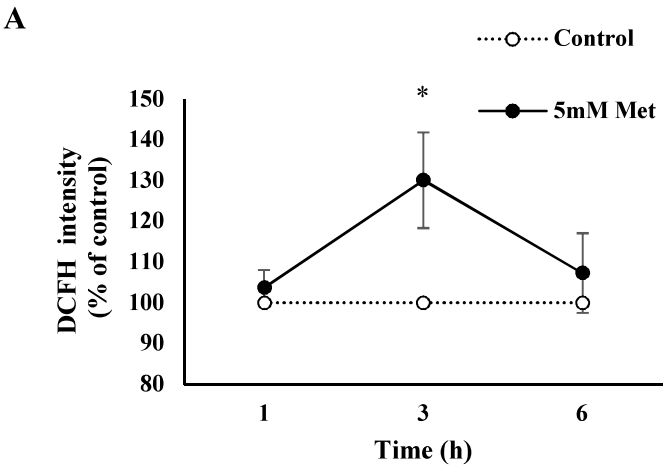


Figure 3. Effect of metformin on E-cadherin expression. (A) Cells were treated with metformin at the concentrations of 1 and 5 mM for 24 h. Total protein was collected and the expression of E-cadherin was analyzed by Western blot analysis. β-actin was used as an internal control. (B) The expression of E-cadherin was shown in quantitative data. The data represented the mean ± standard deviation of at least three experiments. * $P<0.05$ compared to untreated control group. Met: metformin

Metformin-induced ROS production inhibited cell adhesion expression

ROS served as the second intracellular messenger which regulated cell proliferation and cell survival (Ray et al., 2012), while the accumulation of ROS implicated in mediation of apoptosis (Redza-Dutordoir, 2016). Previous study reported that metformin increased ROS production by inhibition of mitochondrial complex I (Lonardo et al., 2015). Therefore, the intracellular ROS levels in metformin treatment were also measured using DCFH-DA fluorimetric assay. The intracellular ROS levels in 5 mM metformin treatment were dramatically increased at 3 h compared to untreated control group (Figure 4A). ROS were reported to modulate either stabilization or destabilization of cell-cell junction-mediated cell-adhesion (Goitre et al., 2012). The effect of metformin-mediated ROS on E-cadherin expression by NAC, ROS scavenger, pretreatment was further examined using Western blot analysis (Figure 4B). HeLa cells were pretreated with 10 mM NAC 1 h prior to 5 mM metformin treatment for 24 h. The levels of E-cadherin expression in NAC pretreatment was completely retrained compared to metformin treatment alone (Figure 4C). Collectively, metformin-mediated ROS production suppressed cell-adhesion expression.



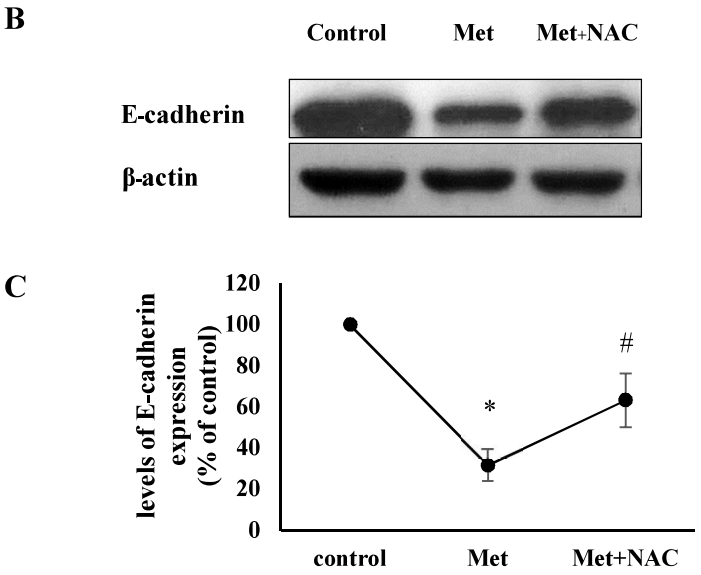


Figure 4. Effect of metformin-induced ROS production on E-cadherin expression. (A) DCFH intensity presented the intracellular ROS production in 5 mM metformin treated cells for 1, 3 and 6 h. (B) The levels of E-cadherin expression in cells treated with 5 mM metformin for 24 h or NAC pretreatment for 1 h followed by 5 mM metformin for 24 h was determined by Western blot analysis. (C) The levels of E-cadherin expression were presented in quantitative data. The data represents the mean ± standard deviation of at least three experiments. * $P<0.05$ compared to control group, # $P<0.05$ compared to metformin treated group. Met: metformin, NAC: Cysteine N-Acetyl Cysteine

DISCUSSION

Our results indicated that metformin inhibited HeLa cell proliferation (Figure 1) through apoptosis induction (Figure 2) in a dose-dependent manner. The results accompanied by impaired E-cadherin expression in metformin treatment at high dose (Figure 3B). The underlying mechanism linking apoptosis induction and cell adhesion impairment was related to the increased ROS production. Metformin-induced ROS production at 3 h (Figure 4A)

could directly trigger mitochondrial-mediating apoptotic cascade at early time point. The results consisted with previous study that metformin-induced ROS production by suppressing mitochondrial respiration rate and depolarization contributed to metformin-induced cell apoptosis (Li et al., 2018). Another way, the increased ROS production in metformin treatment suppressing cell-adhesion expression, prevented by NAC pretreatment, (Figure 4) involved in apoptosis. Our results were agreed with previous evidence that hydrogen peroxide, a major member of ROS, disrupted E-cadherin-catenin interaction in CaCo2 cells monolayer resulting in loss of their integrity though tyrosine kinase-dependent (Basuroy et al., 2002). Previous studies suggested that the loss of cell adhesion by blocking E-cadherin function contributed to an early trigger of cytochrome c releasing from mitochondria to cytosol and activated apoptotic cascades (Galaz, 2005). Similar to other studies, suppression of E-cadherin augmented PARP cleavage and caspase 3 activation (Han et al., 2014). Therefore, our results suggested that metformin-induced cell apoptosis could link to cell adhesion suppression by elevated ROS generation.

CONCLUSION

Metformin has a potential role in anti-proliferation effect by apoptotic induction in HeLa cells. The mechanism of metformin-induced cell apoptosis would be associated with metformin suppressed cell adhesion expression via the increased ROS production. These findings suggested that metformin might be an alternative employment for the clinical treatment of cervical cancer.

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