Metformin, a widely prescribed anti-diabetic drug, also exerts anti-cancer effects in different types of cancers. Although a number of molecular mechanisms have been suggested, the metabolic features underlying metformin’s anti-cancer activity is not fully understood enough. Because cancer cells have been known to prefer inefficient aerobic glycolysis to support their proliferation, it is important to clarify by which metformin affects metabolism to suppress the proliferation of cancer cells. Here, we report the metabolic changes induced by metformin and its relevance to the induction of apoptosis in H4IIE rat hepatocellular carcinoma cells. H4IIE cells were treated with metformin and other reagents in culture media with various nutritional compositions. Glutamine as well as pyruvate enhanced the viability of H4IIE cells in glucose-deprived conditions. Protective effects of glucose and pyruvate were comparable at same concentrations (5 mM). Metformin induced apoptosis irrespective of any nutritional conditions. Glucose consumption and lactate production were stimulated by metformin. Inhibition of glycolysis by 2-deoxyglucose suppressed the metformin-induced lactate production but additively enhanced metformin’s pro-apoptotic effect. These results indicate that metformin does not interfere but accelerate glycolysis. Unexpectedly, the production of reactive oxygen species (ROS) was markedly stimulated by metformin. A potent antioxidant, N-acetylcysteine (NAC) suppressed all pro-apoptotic changes as well as ROS generation induced by metformin. Taken together, metformin does not interfere with glycolysis but promotes apoptosis by enhancing oxidative stress.

Key words metformin; apoptosis; glycolysis; oxidative stress; H4IIE hepatocellular carcinoma cell

INTRODUCTION

Metformin (N,N-dimethylbiguanide) is a widely prescribed hypoglycemic drug for the treatment of Type II Diabetes Mellitus (T2DM). Metformin lowers blood glucose levels through various metabolic changes. Metformin-stimulated AMP-activated protein kinase (AMPK) suppresses hepatic gluconeogenesis but enhances muscular glucose uptake. Metformin also increases insulin receptor expression and receptor tyrosine kinase activity, helps to overcome insulin resistance in T2DM patients. Currently, metformin’s pharmacological significances are recognized for the protection of several diseases and malignancies. Clinical evidences clearly demonstrate a strong correlation between T2DM and the incidence of cancers. On the cellular basis, metformin’s anti-cancer effect might be exerted by blocking cell proliferation or inducing apoptosis. Indeed, metformin affects the AMPK/mammalian target of rapamycin (mTOR) pathway, involved in the control of cell proliferation and protein synthesis. However, the suppression of mTOR by metformin represents merely a line of numerous anti-cancer mechanisms of metformin. Notwithstanding numerous studies about the metformin’s anti-cancer efficacy, our knowledge is strictly limited due to mismatches and diversities different cancer cell types and surrounding environments.

Cancer cells have a distinctive energy metabolism uncommon in normal cells. The concept of cancer cell metabolism was originally established by the Warburg effect, the increase of anaerobic glycolysis even in the presence of oxygen in tumor cells which have mitochondrial dysfunctions. As a result, tumor cells utilize more glucose than normal cells. Thus, the accelerated anaerobic glycolysis is a compensatory mechanism to overcome the mitochondrial respiratory dysfunction in tumor cells. However, it is still controversial whether the failure of mitochondrial respiration is a common feature of various tumor cells because many tumor cells retain normal respiratory activity as well as normal mitochondrial structure. Despite the controversy, the Warburg effect has gained more interests because a series of conventional- or advanced anti-cancer therapies have been not successful. In this context, recent studies have more interests on the metabolic interference by metformin for its anticancer activity. Our previous study reported that glucose-deprived hepatocellular carcinoma cells are more sensitive to metformin. Because liver is the central metabolic organ responsible for systemic energy metabolism, it might have a unique feature in its tumorigenesis compared to other organs. We herein report the effect of metformin on glucose consumption, lactate production, reactive oxygen species (ROS) generation, and their roles in metformin-induced apoptosis of H4IIE rat hepatocellular carcinoma cells

MATERIALS AND METHODS

Cell Culture H4IIE cells were obtained from the Korean Cell Line Bank (Seoul, Korea) and maintained in Dulbecco’s minimal essential medium (DMEM, 5.5 mM glucose) with 10% fetal bovine serum (FBS). At the beginning of the experiments, H4IIE cells were incubated in serum-free DMEM overnight. Cells were washed with Dulbecco’s phosphate-buffered saline (D-PBS) and further incubated in serum- and glucose-free DMEM (GFM) containing different nutrients and test reagents.

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Materials

Cell culture reagents except FBS were purchased from Sigma-Aldrich Chemical Corp. (Sigma, St. Louis, MO, U.S.A.). FBS was obtained from Life Technologies, Inc. (Rockville, MD, U.S.A.). Polyclonal- and monoclonal antibodies against poly ADP ribose polymerase (PARP), cleaved caspase-3, phospho-AMPK, and β-actin were purchased from Cell Signaling Technology (Danvers, MA, U.S.A.). Horseradish peroxidase-conjugated secondary antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). Electrohoresis reagents were obtained from Invitrogen (Carlsbad, CA, U.S.A.). Unless otherwise specified, the other reagents were purchased from Sigma.

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium Bromide (MTT) Assay

Cell viability was analyzed using the MTT assay as previously described. Briefly, H4IIE cells were incubated in D-PBS containing MTT (0.5 mg/mL) for 30 min at 37°C, cells were washed with D-PBS, and the blue-colored formazan product was subsequently solubilized in 0.5 mL of 2-propanol for 20 min. The absorbance of the converted dye was measured at a wavelength of 570 nm.

Lactate and Lactate Dehydrogenase (LDH) Assay

Lactate concentration in the medium was measured with a lactic acid assay kit from Megazyme (Wicklow, Ireland). The amount of intracellular LDH was measured with an assay kit (TaKaRa, Kusatsu, Shiga, Japan). After treatments, cells were collected by a rubber cell scrapper and lysed in a lysis buffer (50 mM Tris–HCl, 1% nonidet P-40, 0.25% sodium deoxycholate, 150 mM NaCl). Equal volume of cell lysate and assay reagent was mixed. After 20 min incubation at room temperature, the absorbance was measured at a wavelength of 492 nm. Protein concentration in each cell lysate was also measured with BCA-protein assay kit (Sigma) to normalize LDH content. The amount of LDH in each cell lysate was represented as a relative unit (RU).

Measurement of Glucose Consumption

Cells were incubated in serum-free DMEM (5.5 mM glucose). After treatments, glucose concentration in medium was measured with glucose assay reagent (Asan Pharm, Whaseong, Korea) based on the glucose oxidase method. The amount of glucose in the culture medium was subtracted from that of DMEM to calculate the glucose consumption.

ROS Measurement

ROS generation within cells was measured with 2′,7′-dichlorodihydrofluorescein diacetate (H2DCFDA). H4IIE cells were grown in 12-well culture plates and treated with reagents. After treatments, H2DCFDA (10 μM) was added to each well and further incubated for 30 min. The supernatant was removed from each well, cell monolayers were dispersed with trypsin–ethylenediaminetetraacetic acid (EDTA) solution. Each volume of cell suspensions were transferred into a black 96-well plate, and the fluorescence intensity of DCF was measured at 485 nm/535 nm for excitation/emission with a multi-well fluorescence reader (Spectrafluor, Tecan, Austria). Results are represented as an RU.

Western Blotting Analysis

After treatment, the cells were lysed in an ice-cold lysis buffer (50 mM Tris–HCl, 1% nonidet P-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM sodium orthovanadate, 1 mM NaF, 1 mM phenylmethylsulfonyl fluoride, 1 mM aprotinin, 1 mM leupeptin, and 1 mM pepstatin A). Equal amounts (10–20 mg) of protein were separated using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 4–12% polyacrylamide gels and transferred onto polyvinylidene difluoride (PVDF) membranes. The membranes were incubated in blocking buffer (5% nonfat dry milk in Tris-buffered saline [TBS]-0.1% Tween-20 [TBS-T]) for 1 h at room temperature, after which...
the membranes were probed with different primary antibodies (at dilutions of 1:1000–1:2000). After a series of washes, the membranes were further incubated with the respective horse-radish peroxidase (HRP)-conjugated secondary antibodies at a dilution of 1:10000. The signal was detected using the enhanced chemiluminescence (ECL) detection system (Intron, Seongnam, Korea).

H33342 Staining The degree of nuclear condensation (a marker of apoptosis) was observed using a cell membrane-permeable DNA-specific fluorescent dye (bisBenzimide H33342 trihydrochloride, H33342). After treatment, the cells were incubated with H33342 (1 mg/mL) for 15 min, then observed under a fluorescent microscope (IX70, Olympus, Japan) and imaged using a digital camera (DP-70, Olympus).

Statistics The experimental results are presented as the mean ± standard error (S.E.). The significance of the differences among groups was determined using Student’s t-test. p < 0.05 was considered statistically significant.

RESULTS

Effects of Nutrients on the Basal Viability and Apoptosis

DMEMs have different compositions of glucose, pyruvate, and glutamine. These formulations are generally designed to maintain the optimal in vitro proliferation of cultured cells. Then we first aimed to examine the effect of each nutrient on the viability of H4IIE cells. Glucose (a major substrate for glycolysis and subsequent mitochondrial respiration), pyruvate or glutamine (substrates for mitochondrial respiration) were added to the serum/nutrients-free GFM. After incubation for 24 h in those defined media, the viability of H4IIE cells was increased 2.6 folds by 1 mM glucose over the control (serum/nutrients-free) group (Fig. 1A). Addition of glucose (5 to 25 mM) did not further increase the viability of cells. Pyruvate (0–25 mM) significantly increased the viability of cells in a dose-dependent manner showing the maximum (2.5 folds of the control) at 5 mM. Glutamine (5 mM) significantly increased the viability (1.5 folds of the control). The formation of apoptotic bodies was accelerated in cells incubated in the nutrient-free culture medium but prevented by the addition of each nutrient (glucose, pyruvate, and glutamine) (Fig. 1B). These results indicate that mitochondria of H4IIE cells can produce enough ATP from pyruvate or glutamine to retain the viability of cells even in glucose-free conditions. From the Western blot analysis, the cleavages of PARP/caspase-3 were enhanced in the control (serum/nutrients-free) but markedly reduced by...
glucose (5 mM) or pyruvate (5 mM) or marginally by glutamine (5 mM) (Fig. 1C). These results indicate that H4IIE cells retain their viability through cytoplasmic glycolysis and/or mitochondrial respiration, like as non-cancerous normal cells.

Effect of Metformin on the Viability of Cells under Different Nutritional Conditions Next, the effect of metformin were examined under different nutritional conditions (Fig. 2A). Viability of metformin-treated cells was significantly reduced in all groups. The loss of viability was highest in the group incubated with 5 mM pyruvate (81% loss vs. the control). Sixty four percent and 41% loss of viability was shown in the 5 mM glutamine and 5 mM glucose-treated groups, respectively. Similar results were found in experiments using 5-aminoimidazole-4-carboxamide ribonucleotide (AICAR) instead of metformin to stimulate AMPK except for the glutamine-containing group. An inhibitor of AMPK (compound C) significantly, but marginally restored the metformin-induced loss of viability (Fig. 2B). It means that AMPK per se is necessary, but not sufficient to mediate metformin’s cytotoxic effect. These results were further supported by Western blot analysis showing the consistent changes of PARP and caspase-3 proteins representing apoptosis (Fig. 2C).

Effect of Metformin on Glucose Consumption, Lactate Excretion, and ROS Generation If cancer cells prefer inefficient glycolysis to produce ATP rather than mitochondria, they require much more glucose than normal cells to compensate less energy efficiency of glycolysis. As a result of the accelerated glycolysis, the basal production of lactate is also elevated higher in cancer cells than normal cells. Excessively produced lactate is excreted to the outside of cells. Thus, we hypothesize that metformin inhibits either glucose consumption or glycolysis and such a metabolic alteration can restrict the viability of H4IIE cells. However, metformin significantly enhanced glucose consumption by 2.5 folds of the control, which was down-regulated to 1.37 folds of the control by compound C (Fig. 3A). The amount of lactate excreted from cell was highly increased by 7.9 folds by metformin, which was suppressed to 4.4 folds of the control by compound C (Fig. 3B). ROS generation was also increased by 1.77 folds by metformin, which was totally suppressed by compound C (Fig. 3C). To verify whether the increased excretion of lactate is the result of metformin-enhanced glycolysis, 2-deoxyglucose (2DG, 2 mM) was added to the culture media (DMEM, 1 mM glucose) together with metformin. 2DG did not affect the basal production of lactate, however, 2DG dramatically blocked the metformin-stimulated production of lactate (1.517 to 0.2273 mmol/L) (Fig. 3D). 2DG additively enhanced the metformin-induced cell death whereas 2DG per se did not affect (Fig. 3E). These results show that metformin enhances the glycolysis, on the contrary to our hypothesis at the beginning of experiments. However, it is still unclear whether the metformin-induced increase of ROS generation is a result of enhanced glycolysis, or other metabolic alterations within cells.

Effect of Antioxidant on the Pro-apoptotic Activity of Metformin If the cytotoxic activity of metformin is mediated by ROS generation and oxidative stress, ROS-scavenging compounds should interfere the metformin’s cytotoxic activity. To confirm this notion, a potent ROS scavenging antioxidant, (N-acetylcysteine, NAC) was treated together with metformin. NAC significantly suppressed metformin-induced glucose consumption (Fig. 4A). Metformin-increased ROS generation as well as metformin-reduced cell viability were significantly interfered by NAC (Figs. 4B, C). Metformin-induced cleavages of caspase-3 and PARP were also suppressed by NAC (Fig.
These results clearly show that metformin-induced cytotoxicity is mediated via ROS generation and oxidative stress.

As described before, it is unclear by which metformin induces ROS generation. Metformin inhibits mitochondrial respiratory complex I and induces membrane depolarization as well. Rotenone is a strong inhibitor of the mitochondrial respiratory complex I. Rotenone-induced incomplete electron transfer within the mitochondrial respiratory chain induces ATP depletion and in turn promotes the formation of ROS and thereby leads to oxidative stress and apoptosis in cells. Thus, we tested whether rotenone mimics metformin’s activity in H4IIE cells. Like as metformin, rotenone markedly stimulated glucose consumption (3.68 folds of the control), which was significantly suppressed by an antioxidant NAC (Fig. 4E). Rotenone significantly increased ROS generation and NAC significantly decreased rotenone-increased ROS generation (Fig. 4F). Rotenone also induced cell death, then significantly suppressed by NAC (Fig. 4G). These results imply that metformin induces ROS generation and oxidative stress, finally leads to the apoptosis of H4IIE cells.

**DISCUSSION**

H4IIE cells survived in the glucose-free conditions if supplemented with pyruvate or glutamine. It means that mitochondria of H4IIE cells can produce ATP enough for sustaining the viability of cells even in the absence of the aerobic glycolysis. In fact, mitochondrial function in most cancer cells is intact. Metformin directly acts on mitochondria to limit respiration, then leads to a compensatory increase in the aerobic glycolysis. Our results showed that metformin promotes glucose consumption and lactate production, suggesting that the aerobic glycolysis is not the direct target of metformin to promote apoptosis of H4IIE HCC cells. Metformin inhibits cancer cell proliferation by suppressing the production of mitochondria-dependent metabolic intermediates required for cell growth. Otherwise, metformin triggers the disorganization of the cristae and inner mitochondrial membrane in several cancer cells and tumors. Excessive production of ROS is a major cause of apoptosis in cancer cells treated with anti-cancer agents. Metformin transiently inhibits colorectal...
cancer cell proliferation as a result of increased ROS production. Mitochondrial complex I is an important source of ROS production. Non-mitochondrial, membrane-bound reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidases (NOXs) also produce ROS. Thus, the precise nature of metformin-induced ROS production should be clarified in terms of the individual role of NOXs as well as mitochondrial respiration. Additionally, the relationship between metformin’s anti-cancer activity and the functional- or structural integrity of mitochondria is further to be elucidated depending on the origin and context of different cancer cell types.

According to the Warburg effect, the basal rate of glucose consumption is higher in tumor cells than normal cells. But little is known about the effect of metformin on glucose consumption in tumor cells. In human lung cancer cells, Calu-1, metformin impairs glucose consumption and survival. In prostate cancer cells, metformin decreases glucose oxidation, the initial step of glycolysis. In our study, metformin increased glucose consumption by 2.5 folds and lactate excretion by 7.9 folds, respectively. It means that aerobic glycolysis is accelerated by metformin because 2DG fully suppressed the metformin-induced lactate production. However, the inhibition of glycolysis per se is not enough to induce cell death because 2DG did not affect cell viability by itself. Instead, 2DG additionally enhanced the metformin-induced death of H4IIE cells. Thus, metformin may not directly interfere glycolysis in metformin-treated H4IIE cells. The ROS-scavenging compound NAC suppressed the metformin-induced cell death and cleavages of caspase-3 and PARP. Metformin-stimulated glucose consumption was also suppressed by NAC. Thus, ROS generation and subsequent oxidative stress should be a direct cause of metformin-induced apoptosis in H4IIE cells.

Although our study confirmed that metformin increases ROS generation as well as glucose consumption, it was still unclear by which metformin can induce ROS generation. As described, mitochondrial complex I is a possible target of metformin. Exactly like as metformin, rotenone markedly stimulated ROS generation and glucose consumption which was again sensitive to NAC. These results raise a question about the interplay between the ROS generation and enhanced glucose consumption. Excessive glucose consumption can result in the massive production of pyruvate if the glycolytic machineries do not have any defects. Then a pool of pyruvate converted into lactate by lactate dehydrogenase A (LDHA). Other pool of pyruvate enters into mitochondria for oxidative phosphorylation, thereby enhancing oxygen consumption and ROS production. Elevated ROS can promote additional glucose consumption by a positive feedback mechanism. Previous studies showed that high glucose induces overproduction of ROS and the exogenous addition of H$_2$O$_2$ stimulates glucose uptake in hepatocytes. Otherwise, if ROS levels exceed a certain threshold, they will impaire mitochondrial respiratory complexes and further stimulate ROS production. NAC lowers intracellular ROS levels, then can suppress ROS-promoted additional glucose consumption. From these results, we hypothesize that the increase of intracellular ROS levels is the downstream of enhanced glucose consumption after metformin treatment.

As metformin induces the deregulated conversion of glucose to pyruvate then excessive amount of pyruvate enters into mitochondria. As a result, it can lead to the massive increase of oxygen consumption and ROS generation. The remaining pyruvate in the cytoplasm is to be converted into lactate, then excreted to the outside of cells. In human lymphoma cells, artificial reduction of LDHA leads to increased mitochondrial oxygen consumption and oxidative stress-induced cell death. Another source of ROS except mitochondria is the ROS-generating NOXs. In cultured vascular cells, hyperglycemia increases diacylglycerol, a strong activator of protein kinase C (PKC), then stimulates ROS production via PKC-dependent activation of NOXs. In HCC, different types of NOXs are expressed, however, little is known about the role of NOXs in the survival or death of HCC. Many evidences have suggested that high glucose induces activation of PKC isoforms. Those findings strongly propose that the high glucose-induced ROS generation by non-mitochondrial NOXs or mitochondrial complex I is initiated by PKC. Our recent experiments found that the inhibition of PKC suppressed metformin-induced increases of ROS production as well as glucose consumption in H4IIE cells (data not shown). However, little is known about on the precise role of metformin in those processes and further to be clarified.

Taken together, we demonstrate that metformin stimulates glucose consumption, ROS generation, and the promotion of apoptosis, which are all sensitive to the antioxidant in H4IIE HCC cells. Thus, we suggest that metformin promotes apoptosis of H4IIE cells by elevated ROS-induced oxidative stress whereas metformin does not interfere with glycolysis.

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Conflict of Interest The authors declare no conflict of interest.

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