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Original Contribution

Metformin induces microRNA-34a to downregulate the Sirt1/Pgc-1α/Nrf2 pathway, leading to increased susceptibility of wild-type p53 cancer cells to oxidative stress and therapeutic agents

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ABSTRACT

Sirtuin 1 (Sirt1) plays an important role in cellular redox balance and resistance to oxidative stress. Sirt1 exhibits oncogenic properties in wild-type p53 cancer cells, whereas it acts as a tumor suppressor in p53-mutated cancer cells. Here, we investigated the effects of metformin on Sirt1 expression in several cancer cell lines. Using human cancer cell lines that exhibit differential expression of p53, we found that metformin reduced Sirt1 protein levels in cancer cells bearing wild-type p53, but did not affect Sirt1 protein levels in cancer cell lines harboring mutant forms of p53. Metformin-induced p53 protein levels in wild-type p53 cancer cells resulted in upregulation of microRNA (miR)-34a. The use of a miR-34a inhibitor confirmed that metformin-induced miR-34a was required for Sirt1 downregulation. Metformin suppressed peroxisome proliferator-activated receptor γ (PPARγ) coactivator-1α (Pgc-1α) expression and its downstream target Nrf2 in MCF-7 cells. Genetic tools demonstrated that the reduction of Sirt1 and Pgc-1α by metformin caused Nrf2 downregulation via suppression of PPARγ transcriptional activity. Metformin reduced heme oxygenase-1 and superoxide dismutase 2 but upregulated catalase expression in MCF-7 cells. Metformin-treated MCF-7 cells had no increase in basal levels of reactive oxygen species but were more susceptible to oxidative stress. Furthermore, upregulation of death receptor 5 by metformin-mediated Sirt1 downregulation enhanced the sensitivity of wild-type p53 cancer cells to TRAIL-induced apoptosis. Our results demonstrated that metformin induces miR-34a to suppress the Sirt1/Pgc-1α/Nrf2 pathway and increases susceptibility of wild-type p53 cancer cells to oxidative stress and TRAIL-induced apoptosis.

Sirtuin 1 (Sirt1), a mammalian NAD^+-dependent histone deacetylase, is involved in diverse cellular processes such as metabolism, cellular redox balance, resistance to oxidative stress, aging, oncogenesis, and cancer development [1,2]. Sirt1 regulates important transcription factors such as p53 [3,4], peroxisome proliferator-activated receptor γ coactivator 1α (Pgc-1α) [5], forkhead homeobox type O (FOXO) proteins [6], and nuclear erythroid factor 2-related factor 2 (Nrf2) [7], which regulates the transcription of pro- and antioxidant enzymes, by which the cellular redox state is affected [1].

Sirt1 plays a critical role in cancer initiation, progression, and drug resistance by blocking senescence and apoptosis and promoting cancer cell growth and angiogenesis [2,8] through inhibition of the tumor suppressor p53, FOXO1, and Ku70-mediated functions [3,4,9,10]. Sirt1 is overexpressed in human breast, colon, non-small-cell lung, and prostate cancer cells [9,11] and a sirtinol- or nicotinamide-specific inhibitor of Sirt1 increased senescence-like growth arrest in human breast, lung, and prostate cancer cells [9,12]. Downregulation of Sirt1 by antisense oligonucleotides inhibited the growth and viability of human prostate cancer [9], induced apoptosis, and enhanced radiation-induced antiproliferative effects in human lung cancer cells [13]. Moreover, pharmacological inhibition of Sirt1 or Sirt1 knockdown induced apoptosis in leukemia stem cells and suppressed growth in vitro and in vivo [14,15]. Sirt1-knockout mice exhibited p53 hyperacetylation, and Sirt1-deficient cells enhanced radiation-induced thymocyte apoptosis, suggesting that Sirt1 can facilitate tumor growth by suppressing p53 function [16]. However, several studies have shown that Sirt1 has tumor-suppressive effects. Activation of Sirt1 by resveratrol inhibited growth of BRCA1-deficient and

Abbreviations: ChIP, chromatin immunoprecipitation; CHOP, C/EBP homology protein; DR5, death receptor 5; HO-1, heme oxygenase-1; IP, immunoprecipitation; miR-34a, microRNA-34a; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Nrf2, nuclear erythroid factor 2-related factor 2; PARP, poly(ADP ribose) polymerase; Pgc-1α, peroxisome proliferator-activated receptor γ coactivator-1α; PPARγ, peroxisome proliferator-activated receptor γ; PPRE, PPAR-responsive element; ROS, reactive oxygen species; SOD2, superoxide dismutase 2; Sirt1, sirtuin 1; TNF, tumor necrosis factor; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand

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$p53^{+/-}$ tumor cells [17] and reduced tumorigenesis in $p53^{+/-}$ mice [18]. Paradoxically, a recent study reported that resveratrol induced apoptosis in wild-type p53 Hodgkin lymphoma cells, which was related to Sirt1 inhibition, p53, and FOXO3a hyperacetylation [6]. These results suggest that Sirt1 has an oncogenic effect in cells expressing wild-type p53 but has a tumor-suppressive effect in mutated p53 cells. Therefore, downregulation of Sirt1 expression or inhibition of Sirt1 activity might be an effective approach to the treatment of cancers harboring wild-type p53.

Nrf2 is a redox-sensitive transcription factor regulating the expression of a battery of cytoprotective genes, including heme oxygenase-1 (HO-1) and antioxidative enzymes such as superoxide dismutase 2 (SOD2) [19]. Constitutive Nrf2 activation in many tumors enhances cell survival and resistance to anticancer drugs [20]. Nrf2 expression is positively regulated by Sirt1 [7], and Nrf2 was shown to be involved in tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) resistance in cancer cells [21]. Downregulation of Sirt1 resulted in induction of death receptor 5 (DR5) and sensitized cancer cells to TRAIL-induced apoptosis [10]. In this study, we investigated the effect of metformin on Sirt1 expression in several cancer cell lines expressing different forms of p53. Our results indicate that metformin upregulates miR-34a to downregulate the Sirt1/Pgc-1α/Nrf2 pathway and increases susceptibility of wild-type p53 cancer cells to oxidative stress. Metformin induces C/EBP homology protein (CHOP) and DR5 expression, enhancing TRAIL-induced apoptosis in wild-type p53 cancer cells.

Materials and methods

Materials

Metformin, TRAIL, and hydrogen peroxide (H_2O_2) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was purchased from USB Corp. (Cleveland, OH, USA), and a lactate dehydrogenase (LDH) release detection kit was obtained from Roche Applied Science (Indianapolis, IN, USA). The plasmid pCMV-β-gal was obtained from Clontech (Palo Alto, CA, USA). Lipofectamine 2000 and nitrocellulose membranes were purchased from Invitrogen (Carlsbad, CA, USA). Oligonucleotide polymerase chain reaction (PCR) primers were custom-synthesized by Bioneer (Seoul, South Korea). A protein assay kit was purchased from Bio-Rad Laboratories (Hercules, CA, USA). Antibodies against Sirt1, Pgc-1, PPARγ, catalase, SOD2, DR5, lamin B1, and β-actin were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-HO-1 antibody was obtained from Calbiochem (San Diego, CA, USA). Antibodies against p53, acetyl-p53, and poly(ADP-ribose) polymerase (PARP), as well as the secondary antibodies horseradish peroxidase (HRP)-linked anti-rabbit and anti-mouse IgG were purchased from Cell Signaling Technologies (Beverly, MA, USA). An antibody against Nrf2 was obtained from Abcam (Cambridge, MA, USA). All other chemicals and reagents were of analytical grade.

Cell culture and treatment

The human breast cancer cell line MCF-7 and human lung adenocarcinoma cell line A549, which contain wild-type p53; MDA-MB-231 cells, which harbor mutant p53; and the p53-null human ovarian cancer line SKOV3 were obtained from the American Type Culture Collection (Rockville, MD, USA). The human colon cancer cell line HCT 116 containing wild-type p53 and p53-knockout HCT 116 cells (HCT 116 $p53^{-/-}$) were a kind gift from Dr. Vogelstein at Johns Hopkins University (Baltimore, MD, USA). All cells were cultured in the appropriate RPMI 1640, Dulbecco's modified Eagle's, or McCoy's 5A medium in a humidified 5% CO_2 incubator at 37 °C in complete medium supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Invitrogen) to 70–80% confluence. Metformin was dissolved in water. TRAIL was dissolved in phosphate-buffered saline containing 0.1% bovine serum albumin. The working concentrations were added directly to serum-free culture medium. Control cells were treated with vehicle only.

Measurement of cell viability and cytotoxicity

MCF-7 cells were cultured in medium containing 10% FBS in 96-well plates at 37 °C. After incubation for 24 h, the growth medium was renewed with serum-free medium and the cells were pretreated with various concentrations of metformin for 24 h, followed by treatment with H_2O_2 or TRAIL for an additional 24 or 48 h at 37 °C, respectively. After treatment, the cells were treated with MTT solution (final concentration, 0.5 mg/ml) for 1 h. The dark blue formazan crystals formed in intact cells were solubilized with dimethyl sulfoxide, and absorbance at 570 nm was measured with a microplate reader (Varioskan; Thermo Electron, Waltham, MA, USA). Cell supernatants were used in lactate dehydrogenase assays, with measurement of absorbance at 490 nm using a microplate reader (Varioskan; Thermo Electron). Percentage cell viability or cytotoxicity was calculated based on absorbance relative to that of vehicle-treated control cells

Luciferase and β-galactosidase assays

Cells were transfected with 0.5 μg of PPRE-luciferase vector and/or 0.2 μg of pCMV-β-gal per well using Lipofectamine 2000. The medium was renewed 6 h after transfection. The transfected cells were treated with metformin (1–5 mM) for 24 h and lysed. The lysed cell preparations were then centrifuged (12,000 rpm, 10 min), and the supernatant was assayed for luciferase and β-galactosidase activities. Relative luciferase activity was determined by normalization to β-galactosidase activity.

Quantitative real-time RT-PCR (qRT-PCR)

After 24 or 48 h of treatment with 1–5 mM metformin, total RNA was isolated from untreated and metformin-treated cells using RNAiso Plus reagent (Takara, Tokyo, Japan) according to the manufacturer's protocol. The concentration and purity of extracted RNA were measured using a Nanodrop instrument. After RNA isolation, cDNA was synthesized using a reverse transcription kit (Promega). Product formation during PCR was monitored continuously using Sequence Detection System software (version 1.7; Applied Biosystems, Foster City, CA, USA) by monitoring increases in reporter dye (SYBR) signals. The mRNA levels of Pgc-1α, Nrf2, CHOP, and DR5 in metformin-treated cells were compared with those in control cells at each time point using the comparative cycle threshold (C_t) method. The following primers were used: human Pgc-1α forward, 5′-AACAGCAGCAGAGACAAATGCACC-3′, reverse, 5′-TGCAGTTCCAGAGAGTTCCACACT-3′; human Nrf2 forward, 5′-TACTCCCAGGTTGCCCACA-3′, reverse, 5′-CATCTACAAACGGGAATGTCTGC-3′; CHOP forward, 5′-CAACTGCAGAGATGGCAGCT-3′, reverse, 5′-CTGATGCTCCCAATTGTTCA-3′; DR5 forward, 5′-GCCCCACAACAAAAGAGGTC-3′, reverse, 5′-GGAGGTCATTCCAGTGAGTG-3′; 18S rRNA forward, 5′-GCTGGAATTACCGCGGCT-3′, and reverse, 5′-CGGCTACCACATCCAAGGAA-3′. The quantity of each transcript was calculated as described in the instrument manual and normalized to the mRNA level of the 18S rRNA housekeeping gene.