Metformin Enhances the Differentiation of Dental Pulp Cells into Odontoblasts by Activating AMPK Signaling

Wei Qin, DDS, PhD,[*†] *Xianling Gao, DDS, PhD,*[*†] *Tao Ma, PhD,*[‡] *Michael D. Weir, PhD,*[†] *Jing Zou, PhD,*[‡] *Bing Song, PhD,*[†] *Zhengmei Lin, DDS, PhD,*[*] *Abraham Schneider, DDS, PhD,*[‡] *and Hockin H.K. Xu, PhD*[†§‖]

Abstract

Introduction: Metformin is a first-line drug for treating type 2 diabetes that regulates the differentiation of mesenchymal stem cells. Its effects on human dental pulp cells (DPCs) remain unknown. This study aimed to investigate the effects of metformin on the proliferation and differentiation of DPCs. **Methods:** A live/dead viability assay kit was used to examine the effects of metformin on the cell viability of DPCs. Cell proliferation was analyzed using a cell counting kit (CCK-8; Dojindo, Tokyo, Japan). Levels of phosphorylated and unphosphorylated adenosine 5′-monophosphate-activated protein kinase (AMPK) were quantified by Western blot analysis in response to metformin and the AMPK signaling inhibitor Compound C (EMD Chemicals, San Diego, CA). The effects of Compound C on the metformin-induced odontoblast differentiation of DPCs were determined by alkaline phosphatase activity assay and von Kossa staining, and the expression of odontoblastic markers was evaluated by reverse-transcription polymerase chain reaction analysis. **Results:** DPCs exhibited mesenchymal stem cell characteristics using flow cytometry. Different doses of metformin were shown to be cytocompatible with DPCs, yielding >90% cell viability. None of the concentrations of metformin up to 50 μmol/L affected cell proliferation. The Western blot assay showed that DPCs express functional organic cation transporter 1, a transmembrane protein that mediates the intracellular uptake of metformin. Metformin significantly activated the AMPK pathway in a dose-dependent manner. In addition, it stimulated alkaline phosphatase activity; enhanced mineralized nodule formation; and increased the expression of odontoblastic markers including dentin sialophosphoprotein, dentin matrix protein 1, runt-related transcription factor 2, and osteocalcin. Moreover, pretreatment with Compound C, a specific AMPK inhibitor, markedly reversed metformin-induced odontoblastic differentiation and cell mineralization. **Conclusions:** This study shows that metformin can induce DPC differentiation and mineralization in an AMPK-dependent manner and that this well-tolerated antidiabetic drug has potential in regenerative endodontics as well as in other regenerative applications. (*J Endod 2017;■:1–9*)

Key Words

AMPK, metformin, odontoblast, proliferation

Significance

Our results indicate that metformin may promote dental pulp tissue healing and repair through AMPK signaling and provide new insights into the therapy of pulpal wounds.

The prevalence of diabetes is increasing globally, and the situation is particularly alarming in Asia. The prevalence of diabetes in China has increased dramatically, from around 1% in 1980 to the most recent estimate of 9.7% according to a nationwide survey (1). Cumulative evidence shows that type 2 diabetes mellitus (T2DM) is associated with aberrant bone formation that can lead to skeletal fractures (2–4). Metformin, a relatively inexpensive and well-tolerated antihyperglycemic biguanide, is widely used by millions of diabetic patients as the first-line treatment for T2DM. As a highly hydrophilic cationic compound, metformin relies on polyspecific cell membrane organic cation transporters (OCTs) of the solute carrier 22A (*SLC22A*) gene family to facilitate its intracellular uptake and action (5). Several studies indicate that metformin has a potential osteogenic effect by promoting the differentiation of mesenchymal stem cells (MSCs) and preosteoblasts. Furthermore, metformin is also able to reverse the deleterious effects of advanced glycation end products on these cells (6–9). Kanazawa et al (10) showed that metformin can induce the differentiation and mineralization of preosteoblasts into osteoblastic cells via activation of the AMPK signaling pathway. Human dental pulp cells (DPCs) share similar gene expression profiles and differentiation capability as other MSCs (11, 12). However, it remains unknown whether metformin is able to induce the odontoblastic differentiation of DPCs.

From the *Department of Operative Dentistry and Endodontics, Guanghua School of Stomatology, Sun Yat-sen University, Guangdong Provincial Key Laboratory of Stomatology, Guangzhou, China; Departments of †Advanced Oral Sciences and Therapeutics, ‡Oncology and Diagnostic Sciences, and §Center for Stem Cell Biology & Regenerative Medicine, University of Maryland School of Medicine, Baltimore, Maryland; and ‖Department of Mechanical Engineering, University of Maryland, Baltimore County, Baltimore County, Maryland.

Address requests for reprints to Dr Zhengmei Lin, Department of Operative Dentistry and Endodontics, Guanghua School of Stomatology, Sun Yat-sen University, 56 Lingyuanxi Road, Guangzhou 510055, China, or Dr Hockin H.K. Xu, Department of Endodontics, Periodontics and Prosthodontics, University of Maryland School of Dentistry, 650 West Baltimore Street, Baltimore, MD 21201, or Dr Abraham Schneider, Department of Oncology and Diagnostic Sciences, University of Maryland School of Dentistry, 650 West Baltimore Street, Baltimore, MD 21201. E-mail address: linzhm@mail.sysu.edu.cn or hxu@umaryland.edu or schneider66@umaryland.edu

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DPCs possess multipotent differentiation potential and the ability to form dentin-pulp–like complexes throughout life. When the dental pulp is confronted by trauma, microbes, or chemicals, a host of inflammatory cytokines are released (13). These insults can also stimulate the underlying progenitor pulp cells to differentiate into odontoblasts, which are capable of secreting dentin matrix proteins as part of reparative dentinogenesis (14). Odontoblasts secrete several collagenous and noncollagenous proteins, such as type 1 collagen, osteopontin, dentin matrix protein 1 (DMP-1), and dentin sialophosphoprotein (DSPP), which are unique biological markers for the odontoblast/osteoblastlike differentiations of DPCs (15, 16). DPCs are potentially superior to other stem cells for regenerative medicine applications including bone tissue engineering. For example, bone marrow MSCs require an invasive procedure to harvest; in contrast, DPCs are easy to harvest from donors including children losing their primary teeth and teenagers having their wisdom teeth removed, which are otherwise discarded as medical waste. Therefore, DPCs are considered to be of great promise for dental repair/regeneration as well as other tissue engineering applications.

Therefore, the present study was designed to examine the effects of metformin on the proliferation and odontoblastic differentiation of DPCs. The role of AMPK signaling in metformin-mediated odontoblastic differentiation was also investigated.

Materials and Methods

Cell Cultures

DPCs were isolated and characterized as described previously (17). Dental pulp tissues were obtained from explants of clinically healthy dental pulp from human adult third molars that were removed from individuals undergoing tooth extraction for orthodontic treatment. The procedure was approved by the Institutional Review Board of the University of Maryland, Baltimore. Briefly, pulp tissues were minced and digested in a solution of 3 mg/mL collagenase type I and 4 mg/mL dispase for 30 to 60 minutes at 37°C. Cell suspension was obtained by passing the digested tissue through a 70-μm cell strainer. The cells were pelleted and seeded in culture dishes and incubated in alpha minimum essential medium supplemented with 20% fetal bovine serum (FBS), 100 units/mL penicillin G, 100 mg/mL streptomycin, and 50 mg/mL ascorbic acid (Sigma-Aldrich, St Louis, MO) at 37°C in 5% CO_2. Nonadherent cells were removed 48 hours after the initial plating. The medium was replaced every 3 days. When the primary culture became subconfluent, after approximately 1 to 2 weeks, cells were collected by trypsinization and subcultured at 5000 cells/cm^2 in growth medium. To analyze cell surface antigen expression, the cells from the second passage were used. The cells were washed and resuspended in phosphate-buffered saline (PBS) supplemented with 3% FBS that contained saturating concentrations (1:100 dilution) of the following reagents: fluorescein isothiocyanate–conjugated antihuman monoclonal antibodies, anti–CD73-phycoerythrin (PE) (BD Biosciences, San Jose, CA), anti—CD90-PE (BioLegend, San Jose, CA), anti–CD105-PE (BioLegend), anti–CD34-PE (BD Biosciences), anti–CD45-PE (BD Biosciences), or anti–human leukocyte antigen-DR (PE) (BD Biosciences) for 1 hour at room temperature in the dark. As a negative control, PE-conjugated nonspecific mouse immunoglobulin G1 (BioLegend) was substituted for the primary antibodies. DPCs from the fourth to fifth passages were used for subsequent experiments.

Before the experiments, DPCs were serum starved in 0.5% fetal calf serum for 18 hours. Different concentrations of metformin were added, and the cells were incubated for the indicated time periods. In the experiments involving the AMPK inhibitor Compound C (EMD Chemicals, San Diego, CA), Compound C was added 1 hour before the addition of metformin, as described later.

Cell Viability

DPCs were seeded in 24-well plates at a density of 3×10^4 cells/well. At 1 and 7 days, cells were stained using a live/dead viability assay kit (Invitrogen Life Technologies, Carlsbad, CA) as we previously described (18). Cells were washed with PBS followed by incubation with the dye. Live cells were stained green with 2 mmol/L calcein AM (Invitrogen Life Technologies), and dead cells were marked red with 4 mmol/L ethidium homodimer-1 (Invitrogen Life Technologies); they were examined using epifluorescence microscopy (Eclipse TE2000-S; Nikon, Melville, NY). The percentage of live cells and the live cell density were calculated as previously described (18). Three random sections were analyzed for each sample.

Cell Proliferation Assays

A cell counting kit (CCK-8; Dojindo, Tokyo, Japan) was used to evaluate cell proliferation at 1, 3, 5, and 7 days as described previously (19). Three replicates in each group were used for this assay ($n = 3$). CCK-8 is based on the Colorimetric Cell Viability Kit I reaction that produces an orange formazan dye in an amount that is directly related to the number of viable cells. The cell proliferative rate was determined via the absorbance at an optical density of 450 nm using a microplate reader (SpectraMax M5; Molecular Devices, Sunnyvale, CA) according to the manufacturer's protocol.

Western Blot Analysis

Cells were harvested and lysed in lysis buffer including 20 mmol/L Na_2PO_4 at a pH of 7.4, 150 mmol/L NaCl, 1% Triton X-100 (Sigma-Aldrich), 1% aprotinin, 1 mmol/L phenylmethysulfonyl fluoride, 10 mg/mL leupeptin, 100 mmol/L NaF, and 2 mmol/L Na_3VO_4. Lysates were centrifuged at 12,000 rpm for 15 minutes. The supernatant was collected, and the protein content was determined using the Bio-Rad protein assay (Bio-Rad, Hercules, CA). Sodium dodecyl sulphate polyacrylamide gel electrophoresis sample buffer (10 mmol/L Tris-HCl, pH = 6.8, 2% sodium dodecyl sulfate, 10% glycerol, and 0.2 mol/L dithiothreitol) was added to the lysates. Lysates were heated to 100°C for 8 minutes, and 20 μg of the total protein was loaded in each well of a 10% sodium dodecyl sulphate polyacrylamide gel electrophoresis gel. Western blot analysis was performed as reported previously (20, 21). The following primary antibodies were used: OCT1 antibody (Sigma-Aldrich), OCT2 antibody (Sigma-Aldrich), OCT3 antibody (Sigma-Aldrich), Phospho-AMPKα (Thr-172) (Cell Signaling Technology, Beverly, MA), total AMPK antibody (Cell Signaling Technology), and glyceraldehyde 3-phosphate dehydrogenase antibody (Santa Cruz Biotech, Santa Cruz, CA).

Alkaline Phosphatase Activity

DPCs were preincubated with 10 μmol/L Compound C for 1 hour and then exposed to 50 μmol/L metformin; this procedure was repeated on day 3. After the treatment, the cells were scraped into cold PBS and then sonicated in an ice bath and centrifuged at 1500*g* for 5 minutes. Then, the alkaline phosphatase (ALP) activity was measured in the supernatant using ALP assay mixtures containing 0.1 mol/L diethanolamine, 1 mmol/L $MgCl_2$, and 10 mg/mL p-nitrophenyl phosphate. After incubation at 37°C for 30 minutes, the reaction was stopped by the addition of NaOH, and the absorbance was measured at 410 nm using a microplate reader (SpectraMax M5).

Reverse Transcriptase Polymerase Chain Reaction and Real-time Quantitative Polymerase Chain Reaction

The expression levels of *ALP*, *DSPP*, *DMP-1*, *runt-related transcription factor 2 (Runx2)*, and *osteocalcin (OCN)* messenger RNA were determined by SYBR (Invitrogen Life Technologies) green