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Metformin protects against retinal cell death in diabetic mice

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ABSTRACT

Retinal degeneration is an early feature of diabetic retinopathy, the major cause of blindness in the developed world. Here we investigated how the widely used antidiabetic drug metformin reduces retinal injury in diabetic mice. Metformin was orally administered to control mice or mice with streptozotocin-induced diabetes. Western blot analysis showed that levels of O-linked β-*N*-acetylglucosamine (O-GlcNAc) transferase (OGT) and other related proteins such as carbohydrate-responsive element-binding protein (ChREBP) and thioredoxin-interacting protein (TXNIP) were significantly increased, and nuclear factor kappaB (NF-κB) and poly (ADP-ribose) polymerase (PARP) were activated in the diabetic retinas or retinal pigment epithelial (RPE) cells exposed to high glucose compared to controls. More importantly, RPE cells exposed to high glucose and treated with thiamet-G had higher levels of those proteins, demonstrating the role of elevated O-GlcNAcylation. Double immunofluorescence analysis revealed increased co-localization of terminal deoxynucleotide transferase-mediated dUTP nick-end labelling (TUNEL)-positive ganglion cells and OGT, ChREBP, TXNIP, or NF-κB in diabetic retinas compared to control retinas. Co-immunoprecipitation analysis showed that interaction between OGT and ChREBP or NF-κB was increased in diabetic retinas compared to control retinas, and this was accompanied by more cell death. Notably, metformin attenuated the increases in protein levels; reduced co-localization of TUNEL-positive ganglion cells and OGT, ChREBP, TXNIP, or NF-κB; and reduced interaction between OGT and ChREBP or NF-κB. Our results indicate that OGT inhibition might be one of the mechanisms by which metformin decreases retinal cell death.

1. Introduction

Diabetic retinopathy (DR) is the most common complication of diabetes and is a major cause of blindness in the western world [1]. Neurodegenerative changes such as neural apoptosis and ganglion cell loss occur in the earliest stages of DR [2]. Although hyperglycemia is the primary pathogenic factor in DR development [3], the mechanisms by which hyperglycemia causes retinal injury remain elusive.

Metformin (*N*,*N*-dimethylbiguanide) is a widely used antidiabetic drug that primarily improves metabolic function by repressing endogenous glucose production and enhancing insulin sensitivity [4,5]. Metformin activates AMP-activated protein kinase (AMPK), and its antidiabetic effect is very likely via this mechanism [6]. Further, metformin protects beta cells by attenuating oxidative stress-induced apoptosis [7]. Although metformin has been used to treat hyperglycemia for decades, the exact molecular mechanisms of its therapeutic action remain unclear. Here, we examined how metformin protects the ganglion cells of the inner retinal layers from cell death in DR.

Modification of intracellular proteins with the O-linked monosaccharide *N*-acetylglucosamine (O-Glc-NAc) is involved in glucose-induced apoptosis [8]. Previous studies showed that carbohydrate-responsive element-binding protein (ChREBP) is a transcriptional regulator of glucose metabolism [9], and ChREBP activity is regulated through multiple post-translational modifications including O-GlcNAc modification. O-GlcNAc modification

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increases ChREBP protein levels and transcriptional activity in the liver [10]. Notably, O-GlcNAc transferase (OGT) facilitates the interaction and O-GlcNAc modification of key insulin signaling regulators [11]. Moreover, thioredoxin-interacting protein (TXNIP), which is transcriptionally regulated by ChREBP [12], is a crucial mediator of glucotoxicity-induced β-cell apoptosis [13]. The mechanism by which glucose activates ChREBP is complex [14], and its role in DR has not yet been elucidated.

Nuclear factor kappaB (NF-κB) has been implicated in inflammatory processes associated with diabetes [15]. The NF-κB family of transcription factors consists of five members: p105/p50, p100/p52, p65 (RelA), c-Rel, and RelB [16]. Many compounds with neuroprotective effects are strongly associated with NF-κB inhibition [17], and NF-κB is an important regulator of programmed cell death [18]. Of note, elevated O-GlcNAc levels enhance NF-κB signaling through increasing the binding of p65/RelA to its target promoters [19], and O-GlcNAc modification of NF-kB is involved in in glucotoxicity [11]. We therefore presumed that O-GlcNAc modification of the p65 could activate NF-κB and affect retinal cell death in diabetic mice. Of additional note, poly (ADP-ribose) polymerase (PARP) is activated by hyperglycemia-induced mitochondrial superoxide, which causes strand breaks in nuclear DNA, leading to PARP activation and hexosamine pathway flux [20] that may increase O-GlcNAc modification and diabetic complications [21].

In this study, we assessed whether metformin decreases retinal neuronal death in diabetic mice, in part driven by post-translational modification of ChREBP and NF-κB, with the aim of developing novel agents for protecting against retinal damage in DR.

2. Materials and methods

2.1. Animals

Diabetes was induced in male C57BL/6 mice (KOATEC, Pyeongtaek, Korea), as previously described [22]. All animal experiments were carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 8023). Metformin was purchased from Enzo Life Sciences (ENZO-ALX-270-432, Farmingdale, NY, USA) and orally given to the mice at 200 mg/kg of body weight. All mice were sacrificed at 2 months after the final injection of 2-deoxy-2-(3-(methyl-3-nitrosoureido)-D-glucopyranose (STZ) or saline. Blood was obtained by tail puncture, and diabetes induction was verified weekly after STZ injection by evaluating blood glucose concentrations using a Precision glucometer (Abbott Laboratories, Alameda, CA, USA). Mice with a blood glucose concentration ≥250 mg/dL were considered diabetic.

2.2. Cell culture and treatments

The ARPE-19 human retinal pigment epithelial (RPE) cell line was obtained from American Type Culture Collection (Manassas, VA, USA), and grown at 37 °C in Dulbecco's modified Eagle medium supplemented with 10% foetal bovine serum (Invitrogen, Carlsbad, CA, USA), 100 g/mL streptomycin, and 100 units/mL penicillin (Invitrogen). Cells were treated with low glucose (LG, 5 mM), high glucose (HG, 25 mM) ± thiamet-G (TMG, 15 μM), HG ± metformin (MET, 80 mM), or HG plus MET ± TMG.

2.3. Metformin administration

Metformin was administered daily by oral gavage. When conventional antidiabetic doses are used in mice, the equivalent dose is 250 mg/kg of body weight/day [5]. Based on a previous report [20], we orally administered 200 mg/kg of body weight/day metformin once a day for 8 weeks after the final STZ or saline injection. Control and diabetic mice were gavaged daily with saline. Blood glucose levels and body weights were measured weekly.

2.4. Antibodies

The following antibodies were used: OGT (sc-74546, Santa Cruz Biotechnology, Santa Cruz, CA, USA; ab96718, Abcam, Cambridge, UK), ChREBP (NB400-135, Novus), TXNIP (sc-166234, Santa Cruz Biotechnology), NF-κB p65 (sc-8008, Santa Cruz Biotechnology), phospho-NF-κB p65 (Ser536) (MA5-15160, Thermo Fisher Scientific, Waltham, MA, USA), PARP (#9632, Cell Signaling, Danvers, MA, USA), cleaved PARP (#5625, Cell Signaling), β-Actin (A5441, Sigma, St. Louis, MO, USA), secondary horseradish-peroxidase-conjugated goat anti-mouse IgG (#31430, Pierce Biotechnology, Waltham, MA, USA), and goat anti-rabbit IgG (#31460, Pierce Biotechnology).

2.5. Western blotting

Protein extraction and western blotting were performed as described previously [22].

2.6. Immunoprecipitation

Immunoprecipitation was performed as described previously [23].

2.7. Immunohistochemistry analysis

Immunohistochemistry was performed on frozen retinal sections (5-μm thick), as described previously [24].

2.8. Immunofluorescence analysis

Immunofluorescence analysis was performed as described previously [23].

2.9. Statistical analysis

Quantitative analyses were performed using ImageJ analysis software (National Institutes of Health, Bethesda, MD, USA) and GraphPad Prism 5 (GraphPad Software, San Diego, CA, USA). Data are representative of three independent experiments and are presented as the means ± standard error of the mean (SEM). The statistical significance of differences was determined using one-way analysis of variance followed by Bonferroni's post hoc analysis to compare groups. Results were considered significant when P was less than 0.05.

3. Results

3.1. Metformin lowers blood glucose levels and promotes weight gain in diabetic mice

Blood glucose levels were significantly increased in diabetic mice compared to control mice (Fig. 1A, $P < 0.0001$). However, metformin treatment significantly lowered the blood glucose levels of diabetic mice compared to diabetic mice not treated with metformin steadily from 1 week to 2 months after diabetes induction, while control mice remained normoglycemic throughout the study (Fig. 1A, $P < 0.0001$). Diabetic mice showed significant weight loss compared to control mice (Fig. 1B, $P < 0.0001$), but metformin moderately increased the body weights of diabetic mice compared to those not treated with metformin (Fig. 1B, $P < 0.05$ or $P < 0.005$).