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TZDs reduce mitochondrial ROS production and enhance mitochondrial biogenesis

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ABSTRACT

Although it has been reported that thiazolidinediones (TZDs) may reduce cardiovascular events in type 2 diabetic patients, its precise mechanism is unclear. We previously demonstrated that hyperglycemia-induced production of reactive oxygen species from mitochondria (mtROS) contributed to the development of diabetic complications, and metformin normalized mt ROS production by induction of MnSOD and promotion of mitochondrial biogenesis by activating the PGC-1α pathway. In this study, we examined whether TZDs could inhibit hyperglycemia-induced mtROS production by activating the PGC-1α pathway. We revealed that pioglitazone and ciglitazone attenuated hyperglycemia-induced ROS production in human umbilical vein endothelial cells (HUVECs). Both TZDs increased the expression of NRF-1, TFAM and MnSOD mRNA. Moreover, pioglitazone increased mtDNA and mitochondrial density. These results suggest that TZDs normalize hyperglycemia-induced mtROS production by induction of MnSOD and promotion of mitochondrial biogenesis by activating PGC-1α. This phenomenon could contribute to the prevention of diabetic vascular complications.

Hyperglycemia in diabetic states increases the risk of diabetic microvascular complications, such as retinopathy, nephropathy and neuropathy, and macrovascular complications, such as cardiovascular disease, stroke and limb amputation. Prospective clinical studies, the Diabetic Control and Complications Trial [1], the UK Prospective Diabetes Study [2] and our Kumamoto Study [3–5] have shown that hyperglycemia strongly contributes to diabetic microvascular complications and macrovascular complications in both type 1 and type 2 diabetes. Intensive blood glucose control in these studies inhibited the initiation and progression of these complications. However, the effect of intensive therapy on prevention of diabetes complications may be limited because of difficulties in maintaining blood glucose concentrations close to the normal range in diabetic patients. Other approaches based on the elucidation of the mechanisms controlling diabetic complications are therefore required to prevent the progression of diabetic complications.

TZDs, including pioglitazone and ciglitazone, are peroxisome proliferator-activated receptor γ (PPARγ) agonists that induce adipogenesis and have insulin-sensitizing and anti-diabetic properties [6]. Recent studies have shown that pioglitazone has the ability to reduce all-cause death, cardiovascular and stroke risk in patients with type 2 diabetes [7]. Pioglitazone resulted in a significantly lower rate of progression of coronary atherosclerosis and slowed progression of carotid artery intima-media thickness compared with glimepiride [8,9]. However, the mechanisms by which TZDs inhibit the progression of atherosclerosis in type 2 diabetes are not well known.

PGC-1α, a transcription coactivator for PPARγ, is a potent stimulator of mitochondrial biogenesis and respiration by regulating nuclear respiratory factors (NRFs). PGC-1α induces strong NRF-1 gene expression. In addition, PGC-1α binds to and coactivates the transcriptional function of NRF-1 on the promoter for mitochondrial transcription factor A (TFAM), a direct regulator of mitochondrial DNA replication/transcription [10].

We previously demonstrated that normalization of the mitochondrial reactive oxygen species (mtROS) production prevented glucose-induced activation of protein kinase C, the formation of advanced glycation end-products and the accumulation of sorbitol in bovine vascular endothelial cells; all of which are involved in the development of diabetic complications [11]. In addition, we also reported that metformin and 5-aminoimidazole-4-carboxamideribonucleoside inhibited the hyperglycemia-induced intracellular ROS and mtROS production, by induction of manganese superoxide dismutase (MnSOD) and promotion of mitochondrial biogenesis by activating the AMP-activated protein

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kinase (AMPK)–PGC-1α pathway [12]. In accordance with our results, it was also reported that PGC-1α positively modulates the expression of the mitochondrial detoxification system, and endothelial cells that overexpress the PGC-1α show reduced accumulation of ROS [13]. Furthermore, PGC-1α-null mice are much more sensitive compared with wild-type mice to the neurodegenerative effects of a neurotoxin, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), which interferes with complex I of the electron transport chain, and results in bioenergetic crisis and ROS activation in the substantia nigra and hippocampus of mice [14].

According to these studies, we hypothesized that TZDs might have the ability to prevent the diabetic complications by suppressing hyperglycemia-induced mtROS overproduction. The present study was designed to investigate whether TZDs inhibit mtROS by activating PGC-1α in human endothelial cells.

Research design and methods

Cells and cell culture. Human umbilical vein endothelial cells (HUVECs) were isolated from human umbilical cord veins by collagenase treatment as described previously [15] and used in passages 1–5. The cells were grown in M199 medium supplemented with 10% fetal bovine serum, 100 μg/ml streptomycin, 100 IU/ml penicillin, 250 ng/ml amphotericin B, 1 mM glutamine, 5 IU/ml heparin and endothelial cell growth supplement at 37 °C within humidified 5% CO_2/95% air. Cultured cells were identified as endothelial cells based on their morphology and by the presence of von Willebrand factor using indirect immunofluorescence microscopy.

Drug treatment. Confluent HUVECs were incubated for 16 h in M199 containing 1% fetal bovine serum (FCS), and then the medium was changed to fresh M199 (1% FCS) containing either 5.6 or 30 mM glucose. In some experiments, HUVECs were incubated with the indicated concentration of pioglitazone (Takeda Pharmaceutical Company Limited, Osaka, Japan) or ciglitazone (Calbiochem, Darmstadt, Germany) for 24 h. During the experiment, the cell viability, evaluated by an Alamar blue assay (Dainippon Pharmaceutical, Osaka, Japan), did not change (data not shown).

Adenoviral vectors. Rat UCP-1 and human MnSOD adenoviral vectors were provided by Dr. M. Brownlee (Albert Einstein College of Medicine, Bronx, NY) [11]. Cells were infected with these adenoviruses 3 days before the experiments. UCP-1 and MnSOD overexpression in HUVECs was confirmed by Western blot analysis, as previously described [12].

Measurement of intracellular ROS. The intracellular formation of ROS was detected using the probe 6-carboxy-2, 7-dichlorodihydrofluorescein diacetate, di (acetoxymethyl ester) (H_2DCF-DA) (C-2938; Molecular Probes, Eugene, OR) after HUVECs were incubated for 24 h under each condition, as previously reported [16]. The ROS concentrations were determined from a standard curve of H_2O_2 (5–50 μM) and were expressed as a percentage of ROS incubated in 5.6 mM glucose.

Fluorescence and light microscopy. To evaluate the direct production of mtROS in HUVECs, we combined the H_2DCF-DA measurements with mtROS specific staining using the reduced MitoTracker Red probe (CM-H_2XRos) (M-7513; Molecular Probes, Eugene, OR) [17]. Briefly, the cells were cultured at 37 °C for 24 h under each condition on glass coverslips in M199 (with 1% FCS) and were then incubated with 0.5 μM CM-H2XRos at 37 °C for 30 min. A confocal laser-scanning microscope (Model FV500; Olympus, Tokyo, Japan), equipped for equifluorescent illumination, was used.

RNA isolation and quantitative RT-PCR analysis of PGC-1α, NRF-1, TFAM and MnSOD mRNA. After incubation for 24 h in each condition, total cellular RNA was isolated from cells using Trizol reagent according to the manufacturer's protocol (Life Technologies, Gaithersburg, MD). To quantify PGC-1α, NRF-1, TFAM and MnSOD transcripts, the Light Cycler System (Roche Molecular Biochemicals, Indianapolis, IN) was used. PCRs were performed using SYBR Green I master mix and specific primers for human PGC-1α: 5′-TCAGTCCT CACTGGTGGACA-3′ and 5′-TGCTTCGTCAAAAACAG-3′; human NRF-1: 5′-CCAGACGACGCAAGCATCAG-3′ and 5′-GGGATCTGGACC AGG CCATT-3′; human TFAM: 5′-TGTTCACAATGGATAGGCAC-3′ and 5′-TCTGGGTTTTCCAAAGCAAG-3′; human MnSOD: 5′-CGACCTG CCC TACGACTACG-3′ and 5′-TGACCACCACCATTGAACTT-3′; and human-actin primers: 5′-TCACCCACACTGTGCCCATCTACGA-3′ and 5′-CAGCGGAACCGCTCATTGCCAATGG-3′. To assess the specificity of the amplified PCR products, after the last cycle, we performed a melting curve analysis and subjected the reaction end-products to electrophoresis in 2% agarose gels, and compared band intensities by imaging of ethidium bromide-stained gels.

DNA isolation and quantitative real-time PCR analysis of mitochondria. Total cellular DNA was isolated using a QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) from HUVECs that were incubated for 24 h under each condition. The specific primer for mtDNA was designed for Complex II (succinate-ubiquinone oxidoreductase) (Human Complex II: 5′-CAAACCTACGCCAAAATCCA-3′ and 5′-GAAATG AATGAGCCTACAGA-3′), and the Light Cycler System (Roche Molecular Biochemicals, Indianapolis, IN) was used to quantify mtDNA.

Mitochondrial quantification. HUVECs were cultured at 37 °C for 24 h under each condition and then prepared for electron microscopy. In brief, the pellets were fixed in 2.5% glutaraldehyde/osmium tetroxide and then sectioned. Thin sections were obtained and viewed by transmission electron microscopy (Hitachi H-7500, Tokyo, Japan). The data were expressed as the mitochondrial density (number of mitochondria per cytoplasmic area). A minimum of 20 cells from each treatment were assessed.

Statistical analysis. The data are expressed as means ± SE. Statistical analysis was performed using unpaired Student's *t*-test or one-way ANOVA followed by the Newman–Keuls multiple-comparison test. *P* values less than 0.05 were considered statistically significant differences.

Results

Effect of pioglitazone and ciglitazone on ROS production in HUVECs

To investigate the effect of pioglitazone and ciglitazone on intracellular ROS production in HUVECs, we used the fluorescent probe H_2DCF-DA. As shown in Fig. 1, H_2DCF-DA-associated fluorescence was significantly increased in the HUVECs after incubation with 30 mM glucose (249.5 ± 12.7% of 5.6 mM glucose) compared with 5.6 mM glucose incubation. Both pioglitazone and ciglitazone suppressed this increased ROS production in a dose-dependent manner (1 μM pioglitazone: 49.0 ± 3.3% of 30 mM glucose; 1 μM ciglitazone: 43.7 ± 3.3% of 30 mM glucose). To elucidate whether TZDs attenuate hyperglycemia-induced ROS production in mitochondria, we examined the mtROS production in HUVECs using reduced MitoTracker Red, a mitochondrion-selective fluorescent probe [17]. As shown in the fluorescence of reduced MitoTracker Red, mtROS was significantly increased in cells incubated with 30 mM glucose compared with cells incubated in 5.6 mM glucose (Fig. 2). As we previously reported, this hyperglycemia-induced-hyperfluorescence was suppressed by the overexpression of UCP-1 or MnSOD, both of which could decrease mtROS production [12]. Moreover, similar to the results obtained using the H_2DCF-DA probe, pioglitazone and ciglitazone also decreased the hyperglycemia-induced fluorescence of reduced MitoTracker Red.

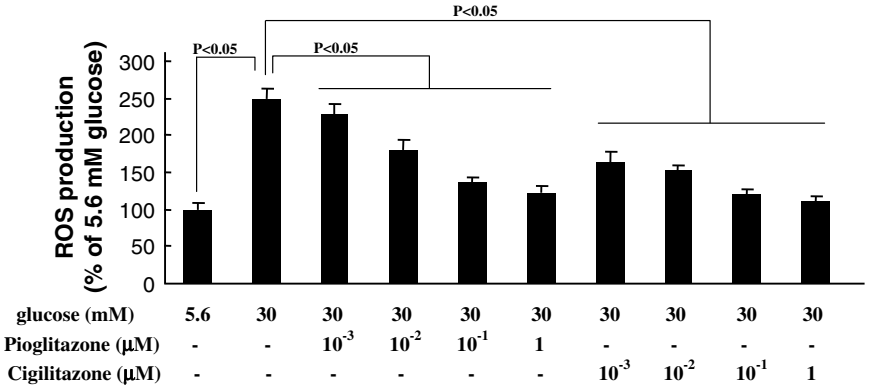

Fig. 1. Effect of TZDs on intracellular ROS production in HUVECs. Cells were incubated under the indicated conditions for 24 h. Intracellular ROS production was quantified using H_2DCF-DA. Results are expressed as values relative to 5.6 mM glucose as 100% (mean ± SE).

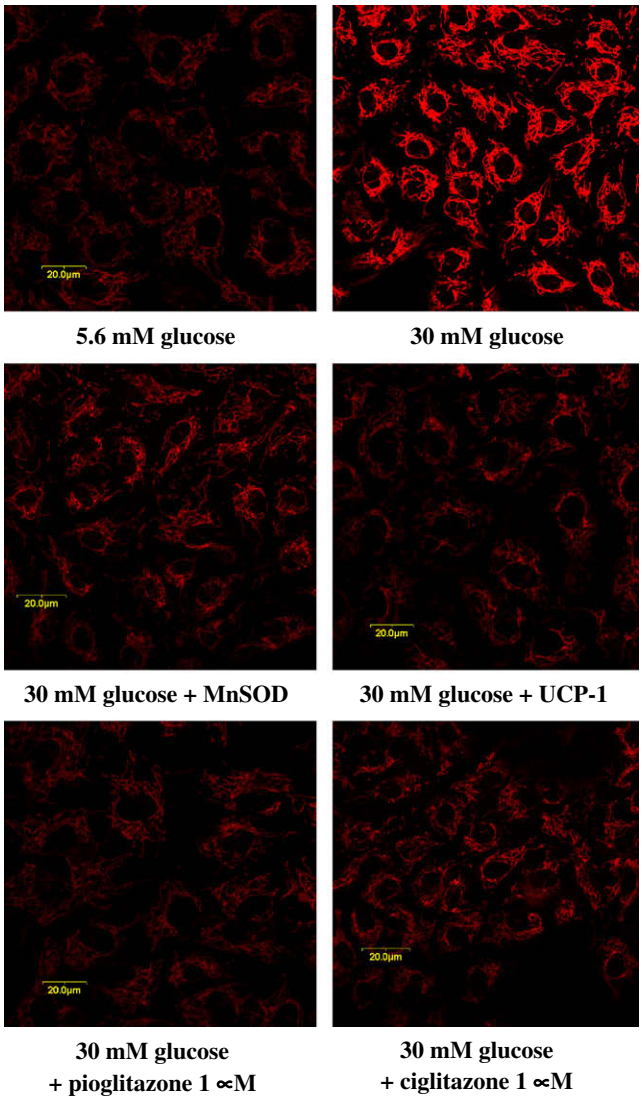

Fig. 2. Fluorescence and light microscopy examinations using MitoTracker Red CM-H2XRos in HUVECs. Mitochondrial ROS production was detected by fluorescence and light microscopy examinations using MitoTracker Red CM-H_2XRos in HUVECs. Cell was infected by adenovirus to overexpress the UCP-1 and MnSOD. The cells were incubated with 1 μM pioglitazone or 1 μM ciglitazone for 24 h.

Effect of TZDs on the expression of PGC-1α and MnSOD in HUVECs

We then investigated the effect of TZDs on the expression of PGC-1α and MnSOD mRNA in HUVECs. As shown in Fig. 3A and B, both pioglitazone and ciglitazone significantly increased PGC-1α and MnSOD mRNA compared with the incubation with 30 mM glucose (1 μM pioglitazone: 143.9 ± 5.8% and 139.8 ± 8.2% of 30 mM glucose; 1 μM ciglitazone: 140.3 ± 12.6% and 38.5 ± 14.5% of 30 mM glucose).

Effect of TZDs on mitochondrial biogenesis in HUVECs

To verify the effect of TZDs on mitochondrial biogenesis, we measured the mRNA levels of NRF-1 and TFAM using quantitative real-time RT-PCR, mitochondrial DNA content by quantitative real-time PCR, and mitochondrial density by transmission electron microscopy. As shown in Fig. 3C and D, the mRNA levels of NRF-1 (1 μM pioglitazone: 123.8 ± 7.0%; 1 μM ciglitazone: 154.0 ± 8.9% of 30 mM glucose) and TFAM (1 μM pioglitazone: 141.5 ± 5.9%; 1 μM ciglitazone: 153.7 ± 8.2% of 30 mM glucose) were significantly increased by pioglitazone and ciglitazone.

As shown in Fig. 4A, mitochondrial DNA content was significantly increased by pioglitazone and ciglitazone compared with 30 mM glucose (1 μM pioglitazone: 121.1 ± 9.1%; 1 μM ciglitazone: 123.0 ± 4.9% of 30 mM glucose). In addition, as shown Fig. 4B and C, transmission electron microscopy revealed that pioglitazone significantly increased the mitochondrial density (number of mitochondria per cytoplasmic area) compared with incubation with 30 mM glucose (1 μM pioglitazone: 144.1 ± 3.1% of 30 mM glucose).

Discussion

In this study, we found that TZDs inhibit hyperglycemia-induced intracellular ROS production and mtROS production in endothelial cells. These phenomena are accompanied by MnSOD induction and mitochondrial biogenesis, which require the induction of PGC-1α.

Many studies have reported that oxidative stress is a potent cause of diabetic complications, including microvascular and macrovascular events [18]. In addition, we previously reported that mtROS plays an important role in the progression of diabetic complications and atherosclerosis, and suggested that drugs which decrease mtROS may prevent these complications [11,16,19–21]. TZDs are clinically beneficial drugs, which not only have an antihy-

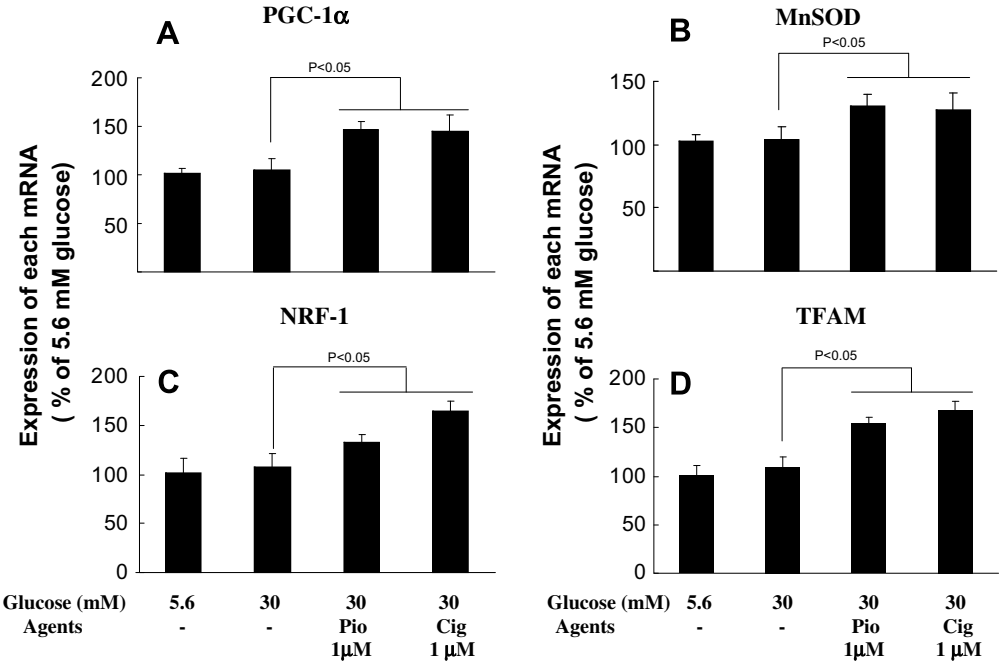

Fig. 3. Effect of TZDs on the expression of PGC-1α, MnSOD, NRF-1 and TFAM mRNA. Cells were treated with 1 μM pioglitazone or ciglitazone for 24 h. The level of PGC-1α (A), MnSOD (B), NRF-1 (C) and TFAM (D) mRNA, were measured by real-time RT-PCR.

perglycemic effect, but may also have pleiotropic effects inhibiting diabetic complications. The present study shows that pioglitazone can decrease mtROS production, and this phenomenon may explain the mechanism of the pleiotropic effects.

We also demonstrated in the present study that the prevention of hyperglycemia-induced mtROS overproduction by pioglitazone was associated with activation of the PGC-1α–MnSOD pathway. In accordance with the observations, we previously reported that metformin also inhibited the hyperglycemia-induced mtROS production by activating the AMPK–PGC-1α pathway [12]. In addition, we confirmed that 1 μM of pioglitazone or ciglitazone more effectively induced MnSOD expression compared with 0.5 mM metformin (1 μM pioglitazone: 120.8 ± 7.2% of 0.5 mM metformin, 1 μM ciglitazone: 119.8 ± 12.5% of 0.5 mM metformin; $P < 0.01$ for both). Therefore, considering these results, TZDs may be more effective for the prevention of diabetic complications than metformin.

It has been reported that pioglitazone induces mitochondrial biogenesis in human subcutaneous adipose tissue *in vivo* [22]. In addition, we have previously reported that metformin can enhance mitochondrial biogenesis [10]. Furthermore, this study also revealed that TZDs have an effect similar to that of metformin in HUVECs. Our findings suggest that the induction of PGC-1α is important for the promotion of mitochondrial biogenesis in HUVECs. On the other hand, hyperglycemia-induced mitochondrial enlargement and dysfunction in primary dorsal root ganglion neurons in diabetic rats were reported to be blocked by thenoyltrifluoroacetone, an inhibitor of mtROS production [23]. Hyperglycemia-induced ROS was also reported to increase mitochondrial DNA oxidative damage and to trigger mitochondrial dysfunction and apoptosis in human retinal vascular endothelial cells [24]. Therefore, inhibition of mtROS by TZDs may prevent mitochondrial dysfunction and apoptosis, and this effect may lead to mitochondrial biogenesis. Mitochondria are the primary source of ATP production and disruption of mitochondrial respiratory function is regarded as a key factor in the development of pathologic changes in the heart and other tissues in diabetes [25–27]. Therefore, the suppression of mtROS may help to prevent diabetic complications by improving mitochondrial function and preventing cell apoptosis in vascular or other tissues in diabetic patients.

We have not shown how TZDs enhance the expression of PGC-1α in HUVECs. The following reasons are possible explanations for the enhanced expression of PGC-1α. First, we previously reported that metformin could induce the activation of PGC-1α expression by activating AMPK [12]; thus AMPK activation may be associated with elevated PGC-1α expression. Second, PPARγ activation was reported to be associated with TZD-induced activation of PGC-1α in brown and white adipocytes from mice [28]. According to this study, PPARγ activation may be associated with TZD-induced activation of PGC-1α in endothelial cells, although the expression of PPARγ was reported to be low in endothelial cells [29]. Finally, it was reported that nitric oxide regulates mitochondrial oxidative stress protection via PGC-1α in endothelial cells [30], and that pioglitazone treatment prevented hypertension and renal oxidative stress both by reducing free-radical production and by increasing nitric oxide production and availability in obese, hypertensive Sprague–Dawley rats [31]. Considering these results, pioglitazone may activate PGC-1α by enhancing nitric oxide production. Further studies are required to clarify these possibilities.

Recently, Nissen et al. reported that rosiglitazone significantly increased the risk of myocardial infarction and the risk of death from cardiovascular causes compared with control groups in the treatment of patients with type 2 diabetes. One reason for this finding could be the differences in lipid metabolism between pioglitazone and rosiglitazone [32]. Further studies are needed to clarify whether not only pioglitazone and ciglitazone, but also all TZDs show an inhibitory effect on hyperglycemia-induced mtROS and promotive effects on mitochondrial biogenesis in vascular endothelial cells.

In conclusion, this study demonstrated that pioglitazone and ciglitazone normalized hyperglycemia-induced intracellular ROS and mtROS production through PGC-1α and MnSOD induction. In addition, pioglitazone and ciglitazone promoted mitochondrial biogenesis. These findings suggest that the drugs, which enhance the PGC-1α–MnSOD pathway and suppress hyperglycemia-in-

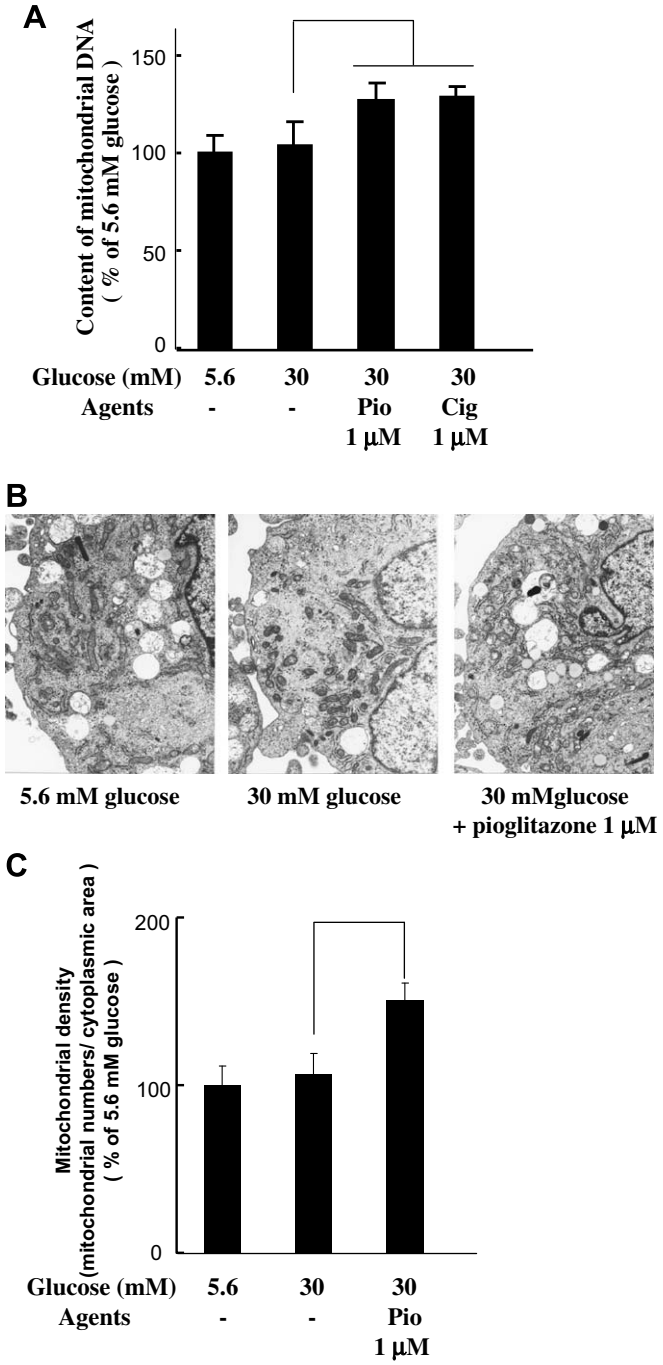

Fig. 4. Mitochondrial DNA content and density. HUVECs were incubated with 1 μM pioglitazone or ciglitazone for 24 h. Mitochondrial DNA content was measured by real-time PCR (A). Mitochondrial density was evaluated using transmission electron microscopy (B). The data were expressed as the mitochondrial density (number of mitochondria per cytoplasmic area) as a percentage of 30 mM glucose (C).

duced mtROS overproduction, may provide a potent therapy for diabetic patients to prevent diabetic macrovascular and microvascular complications.

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