Decrease of peroxisome proliferator-activated receptor delta expression in cardiomyopathy of streptozotocin-induced diabetic rats

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Aims The role of peroxisome proliferator-activated receptor delta (PPARδ) in the development of cardiomyopathy, which is widely observed in diabetic disorders, is likely because cardiomyocyte-restricted PPARδ deletion causes cardiac hypertrophy. Thus, we investigated the effect of hyperglycaemia-induced oxidative stress on the expression of cardiac PPARδ both in vivo and in vitro.

Methods and results We used male Wistar rats to examine the effect of hyperglycaemia on PPARδ expression in streptozotocin-induced diabetic rats, primary neonatal rat cardiomyocytes, and H9c2 embryonic rat cardiomyocytes. PPARδ mRNA (messenger ribonucleic acid) and protein levels were measured using northern and western blotting, respectively. The lipid deposition within the heart section was assessed by oil red O staining. The formation of reactive oxygen species (ROS) and changes in morphology, protein synthesis, and α-actinin content in hyperglycaemic cells were also examined. Inhibitors of ROS production or mitogen-activated protein kinase (MAPK) activation were employed to investigate the possible mechanisms. Cardiomyopathy induced in streptozotocin-diabetic rats was associated with a marked decrease in cardiac PPARδ expression. Also, ROS production, cell size, and protein synthesis were increased while PPARδ expression was reduced in cells exposed to hyperglycaemia in vitro. However, these glucose-induced changes were abolished in the presence of tiron or PD98059 (MEK/ERK inhibitor).

Conclusion Our results suggest that inhibitors of ROS production or MAPK activation are involved in reduction of cardiac PPARδ expression in response to hyperglycaemia.

1. Introduction

The diabetic state can cause cardiac damage in the absence of coronary artery disease. This damage can lead to diabetic cardiomyopathy,1,2 which is characterized by both systolic and diastolic dysfunction due to reduced contractility, prolonged relaxation, and decreased compliance of the myocardium.3,4 Hyperglycaemia is a major factor in the development of diabetic cardiomyopathy.5,6 Hyperglycaemia-induced oxidative stress has been implicated in the onset and progression of diabetic cardiomyopathy.6,7 Also, activation of mitogen-activated protein kinase (MAPKs) plays a key role in the development of diabetic cardiomyopathy.8 The major groups of MAPKs found in cardiac tissue include the p38-MAPK, extracellular signal-regulated kinase (ERK), and c-Jun N-terminal kinase (JNK).8 ERK is strongly activated by mitogen and growth factor, whereas the JNK and p38-MAPK can be activated by various cell stress, including hypertensive shock, metabolic stress, and ischaemia.9–11 The aim of this study was to explore the role of MAPKs in the etiology of diabetic cardiomyopathy.

Peroxisome proliferator-activated receptors (PPARs) are ligand-activated transcriptional factors that regulate expression of genes involved in fatty acid uptake and oxidation, lipid metabolism, and inflammation.12 The three subtypes of PPARs, specifically PPARα, PPARγ, and PPARδ (or PPARδ), have distinct tissue distributions and functions. PPARs form heterodimers with retinoid X receptors, bind to specific hexanucleotide PPAR response elements, and regulate transcription of target genes. Although PPARα and PPARγ are predominantly expressed in liver and adipose

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tissue, respectively. PPARδ is ubiquitously expressed. Moreover, cardiomyocyte-restricted deletion of the PPARδ gene results in cardiac dysfunction, cardiac hypertrophy, and progressive myocardial lipid accumulation. Additionally, a PPARδ-specific agonist inhibited phenylephrine-induced cell hypertrophy and hydrogen peroxide-induced cell apoptosis in cultured cardiomyocytes. Thus, cardiac PPARδ has been proposed as a therapeutic target for treatment of heart disease. However, little is known about the role of PPARδ in the development of diabetic cardiomyopathy.

The present study aims to investigate whether hyperglycaemia-induced cardiac PPARδ expression is altered by the production of reactive oxygen species (ROS) or activation of the MAPK cascade to result in diabetic cardiomyopathy. We examined expression of PPARδ in hearts of rats with a type 1 diabetes-like condition induced by streptozotocin. The mRNA (messenger ribonucleic acid) and protein levels of cardiac PPARδ were examined by northern and western blotting, respectively. Additionally, we tested the effect of ROS on PPARδ expression in neonatal rat cardiomyocytes and H9c2 embryonic rat cardiomyocytes. Intracellular formation of ROS and changes in cell morphology were examined by microscopy. Finally, pharmacological inhibitors were employed to determine the role of ROS and MAPK activation in regulation of PPARδ expression in cardiomyocytes.

2. Methods

2.1 Materials

Streptozotocin, pentobarbital, glucose, phenylephrine, mannitol, trichloroacetic acid, tiron, oil red O and poly-D-lysine were purchased from Sigma-Aldrich, Inc. (Saint Louis, MO, USA). [3H]leucine (9.25 MBq, 250 μCi) was acquired from Amersham Pharmacia Biotech (a division of GE Healthcare, Uppsala, Sweden). 4', 6-diamidino-2-phenylindole (DAPI) and dihydroethidium (DHE) were purchased from Molecular Probes, Inc. (Eugene, Ore). We obtained SB230580 and PD98059 from Tocris Bioscience (Ellisville, MO, USA). Antibodies to PPARδ, actin, α-actinin and IgG-FITC (fluorescein isothiocyanate) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). The plasmid-containing cDNA (complementary DNA) for rat PPARδ was supplied by Ronald M. Evans, PhD, Howard Hughes Medical Institute, Gene Expression Laboratory, The Salk Institute for Biological Studies, La Jolla, CA, USA. The β-actin cDNA was obtained from H.S. Liu, PhD, Department of Microbiology and Immunology, National Cheng Kung University, Tainan, Taiwan.

2.2 Animal model

Eighty male Wistar rats, weighing from 200 to 250 g, were obtained from the Animal Centre of National Cheng Kung University Medical College. Diabetes was induced by intravenous injection of 60 mg/kg streptozotocin. Animals were considered diabetic if they had plasma glucose concentrations of 20 mmol/L or greater in addition to polyuria and other diabetic features. The concentration of plasma glucose was measured by the glucose oxidase method using an analyser (Quik-Lab, Ames, Miles, Inc., Elkhart, IN, USA). All studies were carried out 12 weeks after induction of diabetes. Heart rate and systolic blood pressure were measured non-invasively in conscious rats using the tail-cuff method (MK-2000, Muromachi Kikai Co. Ltd., Tokyo, Japan). Under anaesthesia with pentobarbital, hearts were excised from rats, rinsed with ice-cold phosphate-buffered saline (PBS), gently blotted, and weighed. All animal procedures were performed according to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996), as well as the guidelines of the Animal Welfare Act.

2.3 Tissue preparation and histological analysis

After anaesthesia with pentobarbital, the rats were sacrificed. The rat hearts were perfusion-fixed with 4% paraformaldehyde, 5% sucrose, and 20 mM EDTA (pH = 7.4) for 10 min. The hearts were excised and embedded in optimal-cutting-temperature compound, frozen on dry ice, and then stored at −70°C until sectioning. Serial 15 μm-thick sections were obtained using a sliding microtome (HM525 Cryostat microtome, Microm, Walldorf, Germany). For histological analysis, these sections were collected on gelatin-coated slides, stained with haematoxylin, eosin, and oil red O. Heart sections were stained with oil red O and counterstained with haematoxylin to identify intramyocardial lipid disposition. The images of heart sections were obtained by digital camera and thickness of the left ventricle walls and intraventricular septum were analysed using the Metamorph Image System (Molecular Devices Corp., Downingtown, PA, USA).

2.4 Cell culture and treatment

Primary cultures of neonatal rat cardiomyocytes were prepared by modification of a previously described method. Briefly, the heart tissue from a 1- to 2-day-old Wistar rat was cut into 1-2 mm pieces and digested with trypsin to remove red blood cells. The heart tissue was then digested with 0.25% trypsin and 0.05% collagenase. The dissociated cells were placed in uncoated 100 mm dishes and incubated at 37°C in a 5% CO2 incubator for at least 1 h to remove the non-myocytes. This procedure caused fibroblasts to predominantly attach to the dishes while most of the cardiomyocytes remained unattached. The population of cells enriched in cardiomyocytes was collected and counted. The cells were cultured in Dulbecco/Vogt modified Eagle's minimal essential medium (DME) with 1 mM/L pyruvate, 10% foetal bovine serum (FBS), 100 units/mL penicillin, and 100 units/mL streptomycin. Using this protocol, >95% of the cells were deemed cardiomyocytes as judged by sarcomeric myosin content. On the second day after plating, medium was replaced. Three to 4 days after plating, the cells were exposed to hyperglycaemic conditions as detailed later. Animal handling and disposal were performed in accordance with NIH guidelines.

Embryonic rat-heart-derived H9c2 cells (BCRC 60096) were obtained from the Culture Collection and Research Center of the Food Industry Institute, Hsin-Chiu, Taiwan. The H9c2 cells were maintained in growth medium composed of DMEM supplemented with 10% FBS. Medium was changed every 2 days. Both H9c2 cells and neonatal rat cardiomyocytes were exposed to high glucose in final concentrations of 10, 20, and 30 mM/L in growth medium or 3.5 mM/L glucose as a Control. Cells were incubated with phenylephrine (100 μM/L) or mannitol (30 mM/L) for 24 h. After 24 h exposure, cells were collected and subjected to northern blot or western blot. In immunohistochemical experiments, cells were grown in chamber slides for determination of ROS formation and cellular hypertrophy. Tiron, PD98059, or SB230580 was added 30 min before treatment with glucose at concentrations that are specified in the figure legends.

2.5 Northern blotting

Tissue homogenates were prepared by mechanical homogenization of heart ventricles using a glass/Teflon homogenizer. The concentration of RNA was measured using a spectrophotometer (Pharmacia Biotech, a division of GE Healthcare, Uppsala, Sweden). Total RNA (30 μg) extraction from heart ventricles and cell lysates was completed using TRIzol reagent (Invitrogen Corp., Carlsbad, CA, USA) subjected to electrophoresis through 1.2% formaldehyde-agarose gel. After transferring to Hybond-N membrane (Amersham
International, Buckinghamshire, UK), the RNA was cross-linked to membrane by an UV cross-linker (Stratagene Corporation, an Agilent Technologies company, La Jolla, CA, USA). Probes were labelled with \( {\alpha}^{-32}p \) dCTP using the Medaprime labelling system kit (Amersham International, Buckinghamshire, UK). Blots were pre-hybridized, hybridized, washed twice for 20 min in 2 × sodium saline citrate (SSC)/0.1% sodium dodecyl sulphate (SDS) at room temperature, and washed once for 20 min in 0.1 × SSC/0.1% SDS at 40 °C. Autoradiograms were prepared on Kodak X-ray film (Eastman Kodak Company, Rochester, NY, USA) using a single enhancing screen at −80 °C. Intensity of hybridizing bands on the blot was quantified by scanning densitometry. β-actin was used as an internal standard. Images were analysed using Gel-Pro image analyser software (Media Cybernetics, Inc., Bethesda, MD, USA).

2.6 Western blotting

Protein was extracted from tissue homogenates and cell lysates using ice-cold radioimmunoprecipitation assay buffer [25 mmol/L HEPEs, pH 7.4, 1% (vol/vol) NP-40, 0.1% SDS, 0.5% sodium deoxycholate] supplemented with phosphatase and protease inhibitors (50 μmol/L sodium vanadate, 0.5 mM phenylmethylsulphonyl fluoride, 2 μg/mL aprotinin, and 0.5 μg/mL leupeptin). Protein concentration was determined with Bio-Rad protein assay (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Total proteins (30 μg) were separated by SDS/polyacrylamide gel electrophoresis (10% acrylamide gel) using the Bio-Rad Mini-Protein II system (55 and 130 V). After the removal of primary antibody, the blots were extensively washed with PBS/Tween 20. The blots were then incubated for 2 h at room temperature with the appropriate peroxidase-conjugated secondary antibody dilution in 5% (w/v) skimmed milk powder in PBS. The primary antibody used in western blotting analysis was performed according to the manufacturer’s instructions. Blots were incubated for overnight at 4 °C with an immunoglobulin G polyclonal rabbit anti-mouse antibody (Affinity BioReagents, Inc., Golden, CO, USA) (1:500) in 5% (w/v) skimmed milk powder dissolved in PBS/Tween 20 (0.5% by volume) to bind PPARα in the heart. The blot was incubated with goat polyclonal antibody (1:1000) to bind the actin (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). The intensity of the blot was used to ensure that the amount of protein loaded into each lane of the gel was constant. After the removal of primary antibody, the blots were extensively washed with PBS/Tween 20. The blots were then incubated for 2 h at room temperature with the appropriate peroxidase-conjugated secondary antibody diluted in 5% (w/v) skimmed milk powder dissolved in PBS/Tween 20. Following removal of the secondary antibody, blots were washed according to the technique described previously. The blots were developed by autoradiography using the ELC-western blotting system (Amersham Corp., Braunschweig, Germany). The 49 kDa immunoblot for PPARα and the 43 kDa immunoblot for actin were quantified using a laser densitometer.

2.7 Reactive oxygen species detection

DHE staining was used to quantify ROS production as previously described.7 Cells were incubated with 10 μmol/L DHE for 30 min at 37 °C. The superoxide anion was analysed using an excitation of 518 nm and filter of 585 nm. The fluorescent images were obtained using an Olympus FY1000 laser scanning confocal microscope (Olympus, Tokyo, Japan), and the fluorescent intensity of cells labelled by DHE was counted in each field with image analysis software (FluoViewTM systems, Olympus, Tokyo, Japan).

2.8 Immunofluorescence analysis

To examine the change in morphology, cells were fixed in ice-cold 100% methanol for 10 min. Antibody against α-actinin (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) and DAPI (Probes, Inc., Eugene, Ore) were added at dilutions of 1:400 and 1:150, respectively. In PBS containing 1% bovine serum albumin followed by 1 h incubation at room temperature. The secondary antibody, IgG-FITC (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), was used at a dilution of 1:300 in PBS containing 5% rat serum with 30 min incubation at room temperature. Images were captured by fluorescence microscopy and analysed using the Metamorph Image System (Molecular Devices Corp., Downingtown, PA, USA).

2.9 Incorporation of \([^{3}H]\)leucine

In brief, H9c2 cells treated with glucose, mannitol, or phenylephrine were co-incubated with \([^{3}H]\)leucine (1 μCi/mL) in growth medium for 24 h at 37 °C. After washing with PBS, cells were treated with 10% trichloracetic acid for 30 min at 4 °C to precipitate protein. Precipitates were then dissolved in 0.25 N NaOH and radioactivity was counted using a liquid scintillation counter (LS 5000TA, Beckman Instruments, Inc., Fullerton, CA, USA).

2.10 Flow cytometry

H9c2 cells were treated with 30 mmol/L glucose, 30 mmol/L mannitol, or 100 μmol/L phenylephrine for 24 h and then harvested for flow cytometry. Cells were fixed in 1% paraformaldehyde at 4 °C overnight, washed twice with PBS, and resuspended in PBS. Fixed cells were incubated with antibody to α-actinin for 1 h at 4 °C, which was followed by secondary antibody (IgG-FITC) for 40 min. The stained cells were analysed for their α-actinin content with a FACSscan cytometer (BD Immunocytometry Systems, BD Biosciences, San Jose, CA, USA). Data analysis was done by WinMDI 2.9 software (Joseph Trotter, Salk Institute for Biological Studies, La Jolla, CA, USA), as previously described.21

2.11 Statistical analysis

Data are expressed as means ± SEM for the number of animals in one group or repetitions of cell culture experiments as indicated. Repeated measures analysis of variance was used to analyse changes in plasma glucose and other parameters. Dunnett range post-hoc comparisons were used to determine the source of significant differences. A P-value of 0.05 or less was considered statistically significant.

3. Results

3.1 Cardiac abnormalities and peroxisome proliferator-activated receptor delta in diabetic rats

Administration of streptozotocin resulted in characteristic symptoms of diabetes including hyperglycaemia (28.2 ± 2.1 vs. 6.8 ± 0.5 mmol/L, n = 8, P < 0.001), hypoinsulinemia (1.2 ± 0.8 vs. 159.2 ± 6.1 pmol/L, n = 8, P < 0.001), and decreased body weight gain along with increased food and water intake when compared with age-matched Controls (Table 1). The streptozotocin-induced diabetic rats had lower systolic pressure (96.4 ± 2.6 vs. 119.4 ± 3.3 mmHg, n = 8, P < 0.001) and heart rate (285.3 ± 7.0 vs. 427.5 ± 21.0 beats/min, n = 8, P < 0.001) than the Control rats. The mean heart-to-body weight ratio in streptozotocin-induced diabetic rats was greater than in the Control rats (3.99 ± 0.1 vs. 3.17 ± 0.02 g/kg, n = 8, P < 0.001) (Table 1). Histological analysis showed ventricular hypertrophy, where the sectional areas of the left ventricle walls (0.92 ± 0.04 vs. 1.45 ± 0.03 mm, n = 8, P < 0.01) and interventricular septum (3.48 ± 0.07 vs. 4.56 ± 0.06 mm,
n = 8, P < 0.01) were increased in the hearts of streptozotocin-induced diabetic rats as compared with those of Control rats (Figure 1A) (Table 1). The size and structure of individual cardiomyocytes were larger in diabetic rats than in Control rats (Figure 1A). The mRNA levels of PPARδ were significantly reduced in the hearts of diabetic rats as compared with the Control rats (0.61 ± 0.08 vs. 0.83 ± 0.06, n = 4, P < 0.001) (Figure 1B). Similarly, quantitative analysis revealed that PPARδ protein was significantly lower in the diabetic rats than in the Control animals (0.50 ± 0.03 vs. 0.86 ± 0.02, n = 4, P < 0.01) (Figure 1C). Moreover, intramyocardial lipid deposition was significantly higher in diabetic rats than in Control rats (Figure 1D).

### 3.2 Effect of glucose levels on peroxisome proliferator-activated receptor delta expression in neonatal rat cardiomyocytes and H9c2 cells

We employed primary neonatal rat cardiomyocytes and the rat embryonic cardiomyocyte cell line H9c2 to investigate the effect of high glucose on expression of cardiac PPARδ. Cells were exposed to various concentrations of glucose (10–30 mmol/L) for 24 h to compare to the expression in cells treated with 5.5 mmol/L glucose as a normal

![Figure 1](https://academic.oup.com/cardiovascres/article-abstract/80/1/78/272562)
Control. PPARδ expression in neonatal rat cardiomyocytes was significantly reduced by increasing glucose in a concentration-dependent manner. As shown in Figure 2, a significant reduction in PPARδ mRNA expression was observed in neonatal rat cardiomyocytes exposed to glucose at 30 mmol/L compared with 5.5 mmol/L glucose Controls (0.37 ± 0.04 vs. 0.83 ± 0.04, n = 4, P < 0.001). Additionally, representative immunoblots and quantitative analysis revealed that PPARδ protein was reduced in neonatal rat cardiomyocytes treated with 30 mmol/L glucose for 24 h. Furthermore, the glucose-dependent reduction in PPARδ expression in neonatal rat cardiomyocytes was confirmed by examining mRNA and protein expression in glucose-treated H9c2 cardiomyocytes. Treatment with 30 mmol/L glucose for 24 h significantly decreased PPARδ mRNA and protein expressions to 47.6 ± 4.3 and 39.9 ± 3.8% of control levels, respectively (Figure 2C and D). Because of the limited amount of mRNA and protein obtained from neonatal rat cardiomyocytes, we continued our studies in H9c2 cells to investigate mechanisms of hyperglycaemia-induced PPARδ expression.

3.3 Effects of glucose on reactive oxygen species production and peroxisome proliferator-activated receptor delta expression in H9c2 cells

DHE staining and laser confocal microscopy were used to examine the level of superoxide production in cells. In addition, cells were incubated with FITC-conjugated anti-α-actinin, which is the sarcomere-associated protein. Fluorescence was measured by flow cytometry. Superoxide production was increased 3.3-fold in hyperglycaemia-treated cells (1325.8 ± 32.6, arbitrary units) than in cells grown in control medium (401.3 ± 24.3) (Figure 3A). The α-actinin content was increased (Figure 3B), and PPARδ expression was significantly decreased (Figure 3C and D) in hyperglycaemia-treated cells. However, pretreatment with a superoxide scavenger, tiron (100 μmol/L), inhibited the effects of hyperglycaemia on superoxide production (Figure 3A), α-actinin content (Figure 3B), and PPARδ expression (Figure 3C and D). Treatment with tiron at the maximum dose in normal glucose conditions did not influence the extent of PPARδ expression and α-actinin content.

3.4 Effects of glucose level on cell hypertrophy and peroxisome proliferator-activated receptor delta expression in H9c2 cells

Immunostaining was used to examine the morphological changes induced by hyperglycaemia or 100 μmol/L phenylephrine. It has been reported that phenylephrine is a potent growth factor for cardiomyocytes. Phenylephrine increases the cell size and protein synthesis in cardiomyocytes. To exclude the hyperosmolar effect of hyperglycaemia, we added 30 mmol/L mannitol to control medium to produce identical osmolarity. Immunostaining

Figure 2. Effects of high glucose on peroxisome proliferator-activated receptor delta (PPARδ) expression in neonatal rat cardiomyocytes and H9c2 cells are shown. Cardiomyocytes from neonatal rats (A and B) or the embryonic rat H9c2 cell line (C and D) were cultured with 10–30 mmol/L glucose for 24 h, and then harvested to determine mRNA (messenger ribonucleic acid) (A and C) and protein levels (B and D) by using northern blot and western blot, respectively. All values are presented as mean ± SEM (n = 4 per group). **P < 0.01 and ***P < 0.001 as compared with medium Control.
Figure 3  Effects of tiron on the production of free radicals [reactive oxygen species (ROS)] and peroxisome proliferator-activated receptor delta (PPARδ) expression in hyperglycaemia-treated H9c2 cells. Effects of a ROS-inhibitor (tiron) on the production of ROS (A) and PPARδ expression (B and C) were investigated. H9c2 cells were treated with tiron for 30 min, followed by treatment with 30 mmol/L glucose for 24 h, and then harvested for the following analyses: (A) ROS production was measured using dihydroethidium (DHE) staining, confocal microscopy (at ×200-magnification, scale bar = 100 μm), and an image analysis system. The mean DHE-fluorescence intensity is expressed as mean ± SEM (n = 6 per group). PPARδ mRNA (messenger ribonucleic acid) (B) and protein (C) expression were investigated using northern blotting and western blotting, respectively. (D) The effect of tiron on α-actinin expression in hyperglycaemia-treated cells was determined by flow cytometry using anti-α-actinin-conjugated fluorescein isothiocyanate (FITC). HG, hyperglycaemia (30 mmol/L glucose). All values are expressed as mean ± SEM (n = 4 per group). **p < 0.01 and ***p < 0.001 as compared with Control. ###p < 0.001 as compared with hyperglycaemia-treated cells.
of α-actinin showed an enlargement in cell size in response to hyperglycaemia or phenylephrine (Figure 4A). As described previously,23 we examined the expression of α-actinin in cells using flow cytometry analysis. A distinct rightward shift in the flow cytometry profile was observed in both hyperglycaemia- and phenylephrine-treated cells, which indicated cell hypertrophy. However, α-actinin content was not changed in cells treated with equivalent osmolar medium supplemented with mannitol when compared with control conditions (Figure 4B). Since cellular hypertrophy is defined as an increase in cell size and protein synthesis,24 we characterized the effect of hyperglycaemia on protein synthesis in H9c2 cells using [3H]-leucine incorporation. In comparison with control, protein synthesis was 1.6-fold higher in hyperglycaemic cells and 1.5-fold higher in phenylephrine-treated cells while protein synthesis in mannitol-treated cells remained unchanged (Figure 4C). A marked reduction in PPARδ gene expression, both at the mRNA level

![Figure 4](https://academic.oup.com/cardiovascres/article-abstract/80/1/78/272562/84)

Figure 4 Effects of hyperglycaemia on cell morphology (A and B), protein synthesis (C), and peroxisome proliferator-activated receptor delta (PPARδ) (D and E) expression in H9c2 cells. Cells were treated with 30 mmol/L glucose, 100 μmol/L phenylephrine or 30 mmol/L mannitol for 24 h. The change in cell morphology and fluorescent intensity of fluorescein isothiocyanate (FITC)/α-actinin were measured using fluorescent microscope (A) and flow cytometry (B). Photomicrographs of cell morphology were taken on a fluorescent microscope at ×200-magnification using a digital camera. (C) [3H]leucine incorporation was assessed to measure protein synthesis. All values are expressed as mean ± SEM (n = 8 per group). PPARδ mRNA (messenger ribonucleic acid) (D) and protein (E) levels were measured using northern blot and western blot, respectively. HG, hyperglycaemia (30 mmol/L glucose); PE, phenylephrine. All values are expressed as mean ± SEM (n = 4 per group). ***P < 0.001 as compared with medium Control.
(0.34 ± 0.04 vs. 0.68 ± 0.06, n = 4, P < 0.001) (Figure 4D) and at the protein level (0.46 ± 0.02 vs. 0.78 ± 0.05, n = 4, P < 0.001) (Figure 4E), was observed in cells exposed to hyperglycaemia. The expression of PPARδ was also reduced at both mRNA and protein levels in response to treatment with phenylephrine (Figure 4D and E).

3.5 Effect of mitogen-activated protein kinase inhibitor on the expression of peroxisome proliferator-activated receptor delta in H9c2 treated with glucose

Pharmacological inhibitors were used to clarify the role of MAPK in ROS-induced suppression of PPARδ expression25,26, since previous studies showed that ROS may activate the MAPK pathway to influence cell hypertrophy.8 PPARδ expression in hyperglycaemia-treated cells was not modified by treatment with p38 inhibitor (SB203580) or JNK inhibitor (SP600125) (data not shown). However, treatment with PD98059 to inhibit MEK/ERK activation significantly blocked the reduction in PPARδ expression in hyperglycaemia-treated cells (0.83 ± 0.04 vs. 0.82 ± 0.03 for mRNA level and 0.84 ± 0.03 vs. 0.85 ± 0.03 for protein level; n = 4, P > 0.05; Figure 5A and B). This suggests the involvement of the MEK/ERK pathway in hyperglycaemia- or ROS-induced suppression of PPARδ expression. Furthermore, the significant shift in the flow cytometry profile induced by hyperglycaemia was not observed in the presence of PD98059 (Figure 5C). However, treatment with PD98059 alone did not influence PPARδ expression or cell size.

4. Discussion

In the present study, we found a reduction in PPARδ expression in diabetic rats with cardiomyopathy. High glucose treatment decreased PPARδ expression in neonatal cardiomyocytes and H9c2 cells. Furthermore, immunochemistry and RNA and protein analyses indicated that oxidative stress may suppress PPARδ expression resulting in hypertrophy. However, pretreatment with tiron or PD98059 prevented suppression of PPARδ and cell hypertrophy induced by high glucose. Thus, an activation of the MEK/ERK pathway by hyperglycaemia-induced oxidative stress may be involved in the reduction of cardiac PPARδ expression.

Figure 5 Effects of mitogen-activated protein kinase (MAPK) inhibitors on peroxisome proliferator-activated receptor delta (PPARδ) and α-actinin expression in hyperglycaemia-treated H9c2 cells. H9c2 cells were incubated with 25 μmol/L SB203580 or 12.5 μmol/L PD98059 for 30 min before exposure to 30 mmol/L glucose for 24 h, then harvested to measure mRNA (messenger ribonucleic acid) (A) and protein (B) using northern blot and western blot, respectively. All values are expressed as mean ± SEM (n = 4 per group). (C) The effects of PD98059 on α-actinin expression in hyperglycaemia-treated cells incubated with anti-actinin-conjugated fluorescein isothiocyanate (FITC) were determined by flow cytometry. **P < 0.01 as compared with Control. ***P < 0.01 and ****P < 0.001 as compared with hyperglycaemia-treated cells.
It has been established that PPARδ plays an important role in cardiac function. Also, PPARδ activators attenuate phenylephrine-induced hypertrophy in neonatal cardiomyocytes and reduce ventricular hypertrophy in rats with congestive heart disease. However, little is known about the role of PPARδ expression in the development of diabetic cardiomyopathy. In previous studies, streptozotocin-induced diabetic rats have been used to help elucidate the development of diabetic cardiomyopathy. In this study, we have demonstrated a decrease in PPARδ expression in hearts of streptozotocin-induced diabetic rats. After administration of streptozotocin, rats exhibit cardiac dysfunction and hypertrophy. These results were consistent with previous studies showing symptoms of diabetic cardiomyopathy in streptozotocin-induced diabetic rats. In addition, we found cardiac PPARδ expression was significantly reduced in diabetic hearts at both mRNA and protein levels. Increased lipid contents accumulated in the diabetic heart. Chronic hyperglycaemia has been implicated in the progression of diabetic cardiomyopathy. A recent study demonstrated that cardiomyocyte-restricted PPARδ deletion causes cardiac hypertrophy. Together, these findings indicate that downregulation of PPARδ expression in the diabetic heart can lead to cardiac dysfunction and cardiomyopathy. Thus, reduced PPARδ expression may be involved in hyperglycaemia-induced cardiomyopathy in diabetic rats. In the present study, we cannot exclude the possibility that PPARδ expression was reduced directly by the administration of streptozotocin in rats. Decreased PPARδ expression may cause the accumulation of toxic metabolites in hearts, inducing mitochondrial ROS production. Furthermore, chronic hyperglycaemia may generate a greater amount of ROS to reduce the PPARδ expression. However, the mechanism of action requires further investigation. Hyperglycaemia-induced oxidative stress causes hypertrophy in various types of cells, including cardiomyocytes, renal cells, and pancreatic β-cells. In our study, PPARδ expression was reduced in cardiomyocytes in response to increased glucose concentration in a dose-related manner. Moreover, PPARδ expression, cell size, and protein synthesis were not influenced by high glucose in the presence of the antioxidant, tiron. Thus, the mechanism of ROS-mediated cell hypertrophy in high-glucose-treated cells may involve a reduction of PPARδ expression.

ERK has been shown to have a key role in the mechanism of hypertrophic response. Hypertrophic G-couple receptor agonist, such as phenylephrine, stimulates the ERK pathway in the heart. Also, it was found that in neonatal rat cardiomyocytes, specific inhibition of the ERK pathway reversed phenylephrine-induced protein synthesis and increased cell size. However, the relationship between the ERK cascade and PPARδ expression in cardiomyopathy is still unknown. In this study, we found that hyperglycaemia-induced cell hypertrophy via reduction in PPARδ expression was prevented by pretreatment with a MEK/ERK inhibitor. Cellular stresses are known to upregulate JNK and p38-MAPK, which are thought to be involved in cardiomyocyte apoptosis. However, the apoptotic cells were not observed in our study. Westernmann et al. indicated that the preventive effect of p38 inhibition on diabetes-induced cardiomyopathy in mice is via the reduction of inflammatory cytokines. In our study, pre-treatment with the p38-MAPK inhibitor in glucose-treated cells did not block the reduction of PPARδ expression. Therefore, the ERK cascade can be considered to be involved in hyperglycaemia-induced alterations in PPARδ expression. However, details about the in vivo mechanisms involved require further investigation.

In this study, the reduction of cardiac PPARδ expression is associated with cardiomyopathy in diabetic rats. In conclusion, our results suggest that both ROS production and ERK activation are involved in hyperglycaemia-mediated suppression of PPARδ expression. This finding also supports the key role of PPARδ in the development of diabetic cardiomyopathy.

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Conflict of interest: we declare that we have no conflict of interest.

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