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RESEARCH ARTICLE

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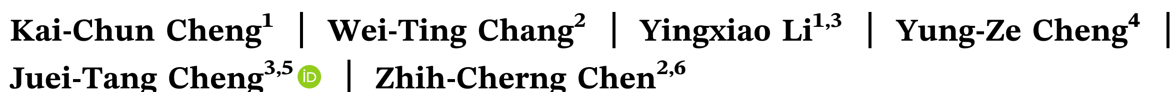
GW0742 activates peroxisome proliferator-activated receptor δ to reduce free radicals and alleviate cardiac hypertrophy induced by hyperglycemia in cultured H9c2 cells



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Abstract
Peroxisome proliferator-activated receptor δ (PPARδ), the predominant PPAR subtype in the heart, is known to regulate cardiac function. PPARδ activation may inhibit cardiac hypertrophy in H9c2 cells while the potential mechanism has not been elucidated. Then, H9c2 cells incubated with high glucose to induce hypertrophy were used to investigate using GW0742 to activate PPARδ. The fluorescence assays were applied to determine the changes in cell size, cellular calcium levels, and free radicals. Western blot analyses for hypertrophic signals and assays of messenger RNA (mRNA) levels for hypertrophic biomarkers were performed. In H9c2 cells, GW0742 inhibited cardiac hypertrophy. In addition, increases in cellular calcium and hypertrophic signals, including calcineurin and nuclear factor of activated T-cells, were reduced by GW0742. This reduction was parallel to the decrease in the mRNA levels of biomarkers, such as brain/B-type natriuretic peptides and β-myosin heavy chain. These effects of GW0742 were dose-dependently inhibited by GSK0660 indicating an activation of PPARδ by GW0742 to alleviate cardiac hypertrophy. Moreover, free radicals produced by hyperglycemia were also markedly inhibited by GW0742 and were later reversed by GSK0660. GW0742 promoted the expression of thioredoxin, an antioxidant enzyme. Direct inhibition of reactive oxygen species by GW0742 was also identified in the oxidant potassium bromate stimulated H9c2 cells. Taken together, these findings suggest that PPARδ agonists can inhibit free radicals, resulting in lower cellular calcium for reduction of hypertrophic signaling to alleviate cardiac hypertrophy in H9c2 cells. Therefore, PPARδ activation can be used to develop agent(s) for treating cardiac hypertrophy.

KEYWORDS
cardiac hypertrophy, GSK0660, GW0742, H9c2 cells, peroxisome proliferator-activated receptor δ (PPARδ)

1 | INTRODUCTION

Cardiac hypertrophy is known to be a response of the heart to extrinsic stimuli, such as hypertension, heart disease, myocardial infarction, and diabetic hypertrophy. Cardiac hypertrophy, as the first phase of cardiovascular disease that induces heart failure, is widely characterized by cell enlargement, which involves physiological and

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pathological hypertrophy.[1] Pathologically, cardiac hypertrophy is often coupled with interstitial and perivascular fibrosis, as well as apoptosis and the release of atrial natriuretic peptides (ANP) and brain/B-type natriuretic peptides (BNP). Upon initiation of cardiac hypertrophy, concentric hypertrophy is the primary phenotype that resists a high afterload and is known as the adaptive phase. Once the cardiac damage progresses, cell length increases, which leads to increased hypertrophy.[2] In cardiac hypertrophy, nuclear factor of activated T-cells (NFAT) is considered to be an important mediator of a number of signal transduction pathways involved in the coordination of pathological stimulation.[3]

In addition, it has been demonstrated that cardiac damage is an adverse effect of diabetes.[4] Left ventricular hypertrophy was found to be associated with diabetes in a multiethnic population.[5] Cardiac hypertrophy and fibrosis are the most frequently observed changes in diabetes.[6] Abnormal metabolites induced by diabetes, such as hyperglycemia and lipid ectopic accumulation in the heart, cause oxidative stress,[7] leading to chronic inflammation, cell apoptosis, vascular endothelial dysfunction, and subsequent basement membrane thickening and cardiac fibrosis, which are widely associated with diabetic cardiomyopathy.[8] Therefore, cardiac hypertrophy has been heavily studied in diabetic disorders, particularly by the use of cardiac cells incubated with high glucose (HG).[9]

Peroxisome proliferator-activated receptors (PPARs) are ligand-activated nuclear receptors that mediate transcriptional regulation by binding to peroxisome proliferator response elements in the regulatory regions of many genes. PPARδ has been identified as the predominant PPAR subtype in cardiac cells.[10] It has been demonstrated that PPARδ activation inhibits phenylephrine-induced cardiac hypertrophy.[11] In addition, the activation of PPARδ has been demonstrated to be effective in angiotensin II-induced cardiac hypertrophy in vitro[12] because cardiac PPARδ deletion in mice results in cardiac hypertrophy.[13]

Recently, the natural product baicalin alleviated cardiac hypertrophy induced by transverse aortic constriction via PPARδ in mice.[14] There is no doubt that PPARδ activation may improve cardiac hypertrophy. However, the potential mechanism(s) for this result remained unclear, although PPARα and PPARδ but not PPARγ were demonstrated to exert antihypertrophic effects via regulation of cardiac metabolism.[15] Therefore, it is of special interest to understand their detailed mechanism(s).

Free radicals are easily associated with cardiotoxicity because the heart is very sensitive to reactive oxygen species (ROS)-induced damage,[16] since the heart is a vital organ with 40% mitochondria[17] by volume to provide cell respiration (aerobic respiration), and the myocardium thus has enzymatic and nonenzymatic systems to neutralize ROS. The role of ROS in the induction of cardiac hypertrophy has been demonstrated.[18] In addition, PPARδ activation protected H9c2 cells from H_2O_2 induced damage.[19] Therefore, it is possible that PPARδ activation may improve cardiac hypertrophy through reduction of ROS, which has not been reported previously.

2 | MATERIALS AND METHODS

2.1 | Materials

GW0742, GSK0660, antioxidant (tiron), and oxidant ($KBrO_3$) were purchased from Sigma-Aldrich. All other reagents were obtained from the supplier as indicated and were at least analytical grade. Antibodies used and their sources are also indicated below.

2.2 | Cell culture

The H9c2 cells (BCRC No. 60096) were cultured following our previous method.[20] In brief, H9c2 cells were maintained in Dulbecco modified Eagle medium (DMEM; pH 7.2; GIBCO-BRL Life Technologies, Gaithersburg, MD) supplemented with 10% fetal bovine serum. The H9c2 cells were plated at a density of 6000 cells/cm2 and allowed to proliferate in the growth medium. After plating, the medium was replaced on the second day. On the next day, the cells were incubated with the testing agent(s) as subsequently described.

Hyperglycemia-treated H9c2 cells were generated by treating the cells with 30 mmol/L glucose for 24 hours, as in our previous study.[20] In brief, the cell culture medium was supplemented with 10% fetal bovine serum. When the cells reached 60% confluence, they were incubated in serum-free medium containing D-glucose (30 mmol/L) for 24 hours, as in our previous study.[20] H9c2 cells exposed to 5.5 mmol/L of D-glucose were used as the control for comparison. The osmolarity variation in any effect of hyperglycemia has been previously ruled out since there are no changes in cells incubated with 24.5 mmol/L. After treatment with HG for 24 hours, the cells were washed twice with the phosphate-buffer solution (PBS) and removed from the culture dishes by trypsinization. The cells were later collected for analysis as described below.

2.3 | Measurement of cardiac hypertrophy

H9c2 cells were arranged on a 24-well plate (Greiner Bio-One, Monroe, NC). Cells were starved for 4 hours

in a serum-free medium before treatment with HG (30 mM) for 48 hours. Briefly, after washing twice with cold PBS, the cells were fixed in 4% paraformaldehyde at room temperature for 15 minutes and washed with PBS containing 2% bovine serum albumin and 0.1% TritonX-100. Cells were stained with rhodamine phalloidin (Invitrogen, Carlsbad, CA) to identify the actin filaments and with 4,6-diamidine-2-phenylindole dihydrochloride (Abcam, Cambridge, MA) to show the nucleus. An entire field of vision was characterized using a microscope (IX71 Olympus, Tokyo, Japan) connected to an imaging system (DP2-BSW, Olympus, Tokyo, Japan). The cell sizes were magnified 200 times and analyzed by the imaging system. Cell surface area size was determined and quantified by imaging to show the complete boundary of individual cells. The results were subsequently expressed as a percentage change in the surface area level in cells based on the analysis using NIH the ImageJ software (Available online: http://imagej.nih.gov/ij/), as described in our previous report.[21]

2.4 | Identification of intracellular superoxide levels

Following the methods described in a previous report,[22] H9c2 cells were seeded in 24-well plates at a density of 7.5×10^3 cells/mL overnight. After starvation for 4 hours in a serum-free medium, the cells were treated with HG at the indicated concentration for an additional 48 hours. For detection of the intracellular superoxide levels, we applied dihydroergotamine from Thermo Fisher Scientific Inc (Rockford, IL) to react with intracellular superoxide ions at 37°C for 30 minutes. An entire field of vision was characterized using a fluorescence microscope (IX71 Olympus) connected to an imaging system (DP2-BSW). The results were subsequently expressed as a percentage of the intracellular superoxide level in the cells based on the analysis using the NIH the ImageJ software (available online: http://imagej.nih.gov/ij/), as described in our previous report.[21]

2.5 | Real-time reverse transcription-polymerase chain reaction

Similar to our previous method,[21] the messenger RNA (mRNA) expression levels of each signal were determined. In brief, total RNA was extracted from the cell lysates with TRIzol reagent (Invitrogen, Carlsbad, CA). Total RNA (200 ng) was reverse transcribed into complementary DNA with random hexamer primers (Roche Diagnostics, Mannheim, Germany). All polymerase chain reaction (PCR) experiments were performed using a LightCycler (Roche Diagnostics GmbH). The concentration of each PCR product was calculated relative to a corresponding standard curve. The relative gene expression was subsequently indicated as the ratio of the target gene level to that of β-actin. The primers for BNP, myosin heavy chain (MHC), and β-actin are listed as follows:

BNP F: 5′-GTCAGTCGCTTGGGCTGT-3′;
BNP R: 5′-CCAGAGCTGGGGAAAGAAG-3′;
β-MHC F: 5′-CATCCCCAATGAGACGAAGT-3′;
β-MHC R: 5′-GGGAAGCCCTTCCTACAGAT-3′;
β-actin F: 5′-CTAAGGCCAACCGTGAAAAG-3′;
β-actin R: 5′-GCCTGGATGGCTACGTACA-3′.

2.6 | Nuclear extraction

We performed the extraction of the nuclear fraction according to our previously described method[23] using a CNMCS Compartmental Protein Extraction Kit (BioChain Institute, Inc, Hayward, CA). Briefly, H9c2 cells were collected and mixed with ice-cold lysis buffer (2 mL per 20 million cells). The cell mixture was passed through the needle base 50 to 90 times to disrupt the cell membranes and to release the nuclei from the cells. The degree of cell membrane disruption and the release of nuclei were monitored with a microscope. The mixture was then centrifuged at 15 000 × g at 4°C for 20 minutes. The supernatant, which contains cytoplasmic proteins, was removed and saved in a separate tube. The pellet was resuspended in ice-cold wash buffer (4 mL per 20 million cells), and the suspension was rotated at 4°C for 5 minutes, followed by centrifugation at 15 000 × g at 4°C for 20 minutes. The supernatant was then removed and ice-cold nuclear extraction buffer (1 mL per 20 million cells) was added to the pellet. After rotating at 4°C for 20 minutes, the suspension was centrifuged at 15 000 × g at 4°C for 20 minutes. The supernatant, which contained nuclear proteins, was removed and saved for future studies.

2.7 | Western blot analysis

We used ice-cold radioimmunoprecipitation assay buffer to extract the proteins from rat heart homogenates or cell lysates. Western blot analysis was subsequently performed according to our previous method.[21] The target antigens from the protein extracts were detected using primary antibodies specific for calcineurin (Sigma-Aldrich, St. Louis, MO), NFAT3 (Thermo-Fisher Scientific), or £]-actin (Sigma-Aldrich) and histone H3 (Santa Cruz, Dallas, TX). The bound primary antibodies were subsequently hybridized to horseradish peroxidase-conjugated goat anti-rabbit

or anti-mouse IgGs (Calbiochem, San Diego, CA), and the immunoreactive bands were developed with a chemiluminescence kit (PerkinElmer, Waltham, MA). The optical densities of the bands for calcineurin (18 kDa), NFAT3 (100 kDa), histone H3 (15 kDa), and β-actin (43 kDa) were quantified as described in our previous report.[24]

2.8 | Measurement of intracellular calcium concentrations

The changes in the intracellular calcium concentration $[Ca^{2+}]i$ were detected using the fluorescent probe fura-2. Cells were placed in a buffered physiological saline solution as described previously,[20] to which 5 μM of fura-2 was added. The fluorescence was continuously recorded using a fluorescence spectrofluorometer (Hitachi F-2000). The intracellular calcium levels $[Ca^{2+}]i$ was calculated automatically. Values of $[Ca^{2+}]i$ were later determined and the background measured in unloaded cells was subtracted from all measurements according to our previous report.[20]

2.9 | Statistical Analysis

The results are presented as the mean ± SEM from the indicated sample size (*n*) in each group. Statistical analysis was performed using one-way analysis of variance followed by Tukey's post hoc analysis to compare the difference. $P < 0.05$ was considered significant.

3 | RESULTS

3.1 | GW0742 alleviates cardiac hypertrophy induced by HG in H9c2 cells

Cardiac hypertrophy has been identified in H9c2 cells incubated with HG (30 mmol/L), as shown in Figure 1A. Cell size was markedly extended compared with the normal group. GW0742 inhibited the cell size of H9c2 cells in a dose-dependent manner (Figure 1A). Mediation of PPARδ has also been characterized in H9c2 cells treated with GW0742 using Western blot analysis (Figure 1B). A decrease of PPARδ expression level was observed in HG-treated H9c2 cells showing hypertrophy. GW0742 reversed the PPARδ expression level in the same dose-dependent way (Table 1). In addition, the increased expression of hypertrophic signals, such as calcineurin (Figure 1C) and nuclear NFAT (Figure 1D), were also reduced by GW0742 in the same fashion (Table 1). Moreover, the mRNA levels of hypertrophic biomarkers, both BNP and β-MHC stimulated by hyperglycemia, were also attenuated by GW0742 in the same way (Table 1). Therefore, cardiac hypertrophy induced by hyperglycemia established in H9c2 cells was alleviated by GW0742 via recovery of the decreased level of PPARδ.

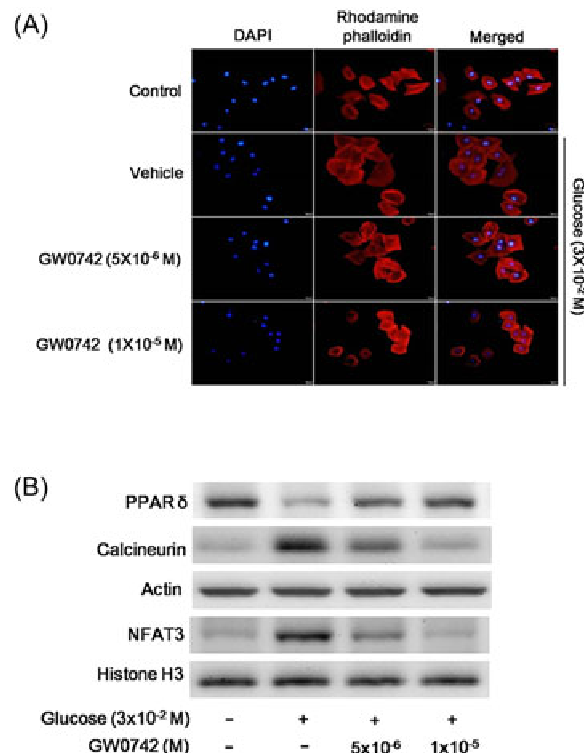

FIGURE 1 Effects of GW0742 on cardiac hypertrophy induced by hyperglycemia in cell line H9c2. Cells incubated with GW0742 at the indicated concentration were used to assay the cell size, (A) and the changes in hypertrophic signals (B). Each one shows the representative response and the quantified data are indicated in Table 1. NFAT, nuclear factor of activated T-cells; PPARδ, peroxisome proliferator-activated receptor δ

3.2 | GSK0660 inhibits the effects of GW0742 in H9c2 cells

GW0742 has been demonstrated to inhibit ROS unrelated to PPARδ activation.[25] Thus, we applied the specific antagonist GSK0660 to identify the mediation of PPARδ activation in GW0742-induced effects. In cultured H9c2 cells, at the dose effective to block PPARδ, GSK0660 inhibited the effects of GW0742 as shown in Figure 2 and Table 2. Therefore, the GW0742-induced effects as described above were primarily produced through PPARδ activation in H9c2 cells.

3.3 | Role of free radicals in the effectiveness of GW0742 produced in H9c2 cells

Cardiac hypertrophy has been shown to be associated with exposure to free radicals and/or ROS.[26] The development

TABLE 1 GW0742 attenuated the hyperglycemia-increased hypertrophic signals in H9c2 cells

Contents	Control	Glucose (3×10^{-2} M)	Glucose (3×10^{-2} M) + GW0742 (5×10^{-6} M)	Glucose (3×10^{-2} M) + GW0742 (1×10^{-5} M)
Relative area level (fold change)	1.00 ± 0.11	3.17 ± 0.29**	1.46 ± 0.05*,***	0.91 ± 0.09
Relative mRNA of BNP/β-Actin	1.00 ± 0.00	2.11 ± 0.12**	1.60 ± 0.07*,***	1.25 ± 0.05
Relative mRNA of β-MHC/β-actin	1.00 ± 0.00	1.98 ± 0.04**	1.56 ± 0.05*,***	1.29 ± 0.05
$[Ca^{2+}]_i$ (fold change)	1.00 ± 0.022	1.87 ± 0.15**	1.29 ± 0.13*,***	1.01 ± 0.07
Ratio of PPARδ/ β-actin protein	0.67 ± 0.05	0.26 ± 0.02**	0.44 ± 0.03*,***	0.62 ± 0.04
Ratio of calcineurin/β-actin protein	0.21 ± 0.04	0.83 ± 0.06**	0.47 ± 0.06*,***	0.27 ± 0.03
Ratio of NFAT3/Histone H3 protein	0.24 ± 0.02	0.69 ± 0.05**	0.44 ± 0.05*,***	0.28 ± 0.03

Abbreviation: BNP, brain/B-type natriuretic peptides; mRNA, messenger RNA; MHC, myosin heavy chain; NFAT, nuclear factor of activated T-cells; PPARδ, Peroxisome proliferator-activated receptor δ.
***$P < 0.05$ varied with the vehicle-treated group under high glucose.
Values (mean ± SEM) were obtained from six samples and/or determinations per group. Effects of GW0742 at the indicated concentrations under high glucose (30 mM) were compared with that under normal medium (control). *$P < 0.05$ or **$P < 0.01$ are significantly different from the control.

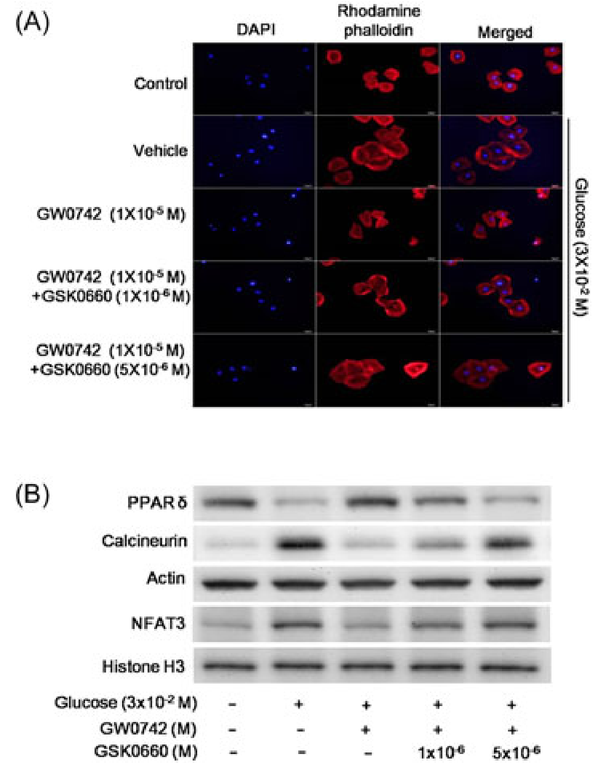

FIGURE 2 Effects of PPARδ blocker GSK0660 on the action of GW0742 in the cell line H9c2. Cells incubated GSK0660 at the indicated concentration before addition of GW0742 were used to assay the cell size (A) and the changes in hypertrophic signals (B). Each one shows the representative response and the quantified data are indicated in Table 2. NFAT, nuclear factor of activated T-cells; PPARδ, Peroxisome proliferator-activated receptor δ

of diabetic cardiomyopathy is associated with oxidative stress from elevated ROS production and/or decreased antioxidant defense.[27] Abnormal metabolites induced by diabetes, such as hyperglycemia in the heart, cause ROS.[7] Therefore, a HG-induced cardiac hypertrophy model has been established in H9c2 cells.[28] PPARδ activation appears to exert most of its direct and indirect inhibitory effects on ROS production in the cardiovascular system.[29] Therefore, we were interested in understanding the role of ROS and/or free radicals in the influence of GW0742 observed in H9c2 cells.

Similar to the previous report,[30] free radicals, including superoxide and ROS, were markedly increased by HG in H9c2 cells as shown in Figure 3A. GW0742 attenuated the increased free radicals in a dose-dependent manner (Table 2). Moreover, the expression of thioredoxin 2 (Trx2), a mitochondrial antioxidant, has been demonstrated to diminish in H9c2 cells by hyperglycemia.[31] Our results are in keeping with these findings, as shown in Figure 3B.

TABLE 2 GW0742-induced actions were inhibited by GSK0660 through blockade of PPARδ in H9c2 cells

Contents	Control	Glucose (3×10^{-2} M)	Glucose (3×10^{-2} M) + GW0742 (1×10^{-5} M) + Vehicle	Glucose (3×10^{-2} M) + GW0742 (1×10^{-5} M) + GSK0660 (1×10^{-6} M)	Glucose (3×10^{-2} M) + GW0742 (1×10^{-5} M) + GSK0660 (5×10^{-6} M)
Relative area level (fold change)	1.00 ± 0.09	2.17 ± 0.18**	0.83 ± 0.11##	1.47 ± 0.16*#	1.97 ± 0.14**
Relative mRNA of BNP/β-actin	1.00 ± 0.00	1.97 ± 0.04**	1.22 ± 0.05##	1.54 ± 0.05*#	2.03 ± 0.05**
Relative mRNA of β-MHC/β-actin	1.00 ± 0.00	1.98 ± 0.02**	1.10 ± 0.06##	1.49 ± 0.05*#	1.95 ± 0.05**
$[Ca^{2+}]_i$ (fold)	1.00 ± 0.06	1.63 ± 0.15**	0.90 ± 0.05##	1.33 ± 0.14*#	1.52 ± 0.13**
Ratio of PPARδ/ β-actin protein	0.90 ± 0.05	0.42 ± 0.05**	0.89 ± 0.09##	0.68 ± 0.07*#	0.44 ± 0.06**
Ratio of calcineurin/β-actin protein	0.38 ± 0.04	0.96 ± 0.06**	0.45 ± 0.03##	0.70 ± 0.04*#	0.89 ± 0.07**
Ratio of NFAT3/Histone H3 protein	0.41 ± 0.04	0.87 ± 0.04**	0.48 ± 0.04##	0.62 ± 0.04*#	0.81 ± 0.03**

Abbreviation: BNP, brain/B-type natriuretic peptides; mRNA, messenger RNA; MHC, myosin heavy chain; NFAT, nuclear factor of activated T-cells; PPARδ, Peroxisome proliferator-activated receptor δ.
Values (mean ± SEM) were obtained from six samples and/or determinations per group. Effects of GW0742 at the indicated concentrations under high glucose (30 mM) were compared between the pretreatment with GSK0660 or not. *$P < 0.05$ or **$P < 0.01$ are significantly different from the control. #$P < 0.05$ or ##$P < 0.01$ versus GSK0660 (5×10^{-6}M)-treated group under high glucose

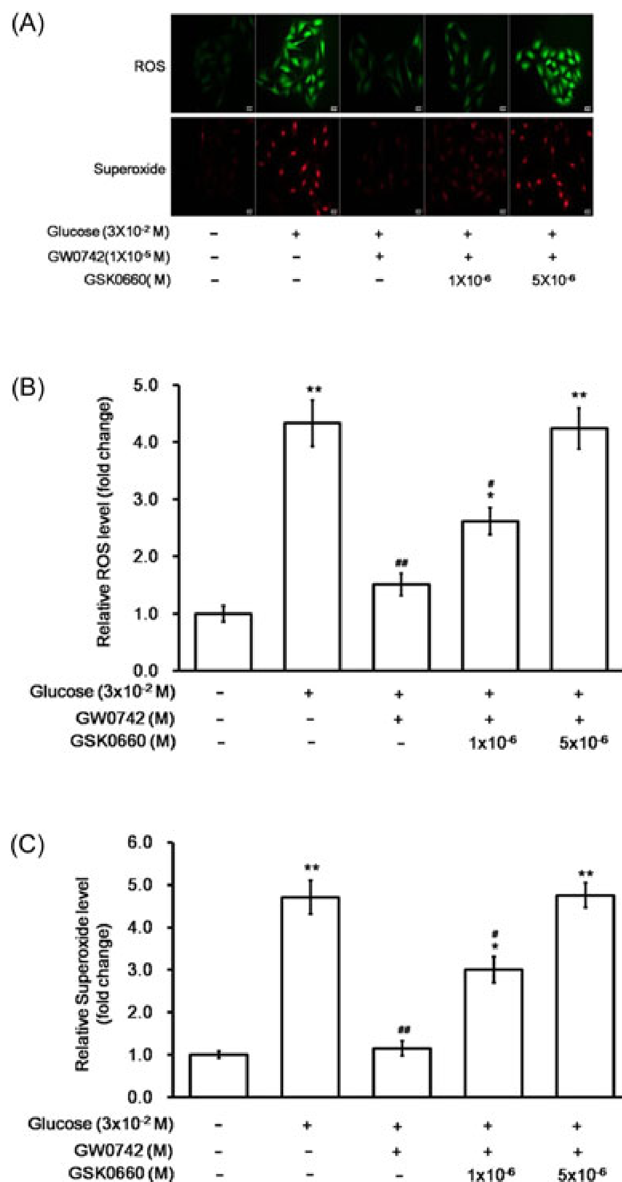

FIGURE 3 GW0742 inhibits the free radicals produced by hyperglycemia in H9c2 cells. The representative response indicates the formation of free radicals, both ROS and superoxide, produced by hyperglycemia in cells (A). The effect of GW0742 was dose-dependently reduced by GSK0660 at the indicated concentrations, compared with the vehicle-treated control. Quantified data regarding the changes in ROS (B) or superoxide (C) are also indicated. Each column shows the mean ± SEM (n = 6). *$P < 0.05$ or **$P < 0.01$ are significantly different from the vehicle-treated control (first column). #$P < 0.05$ or ##$P < 0.01$ are significantly different from the vehicle-treated under high glucose group (the second column). ROS, reactive oxygen species

Interestingly, GW0742 enhanced the gene expression of Trx2, both at the mRNA and protein levels, and this action was blocked by GSK0660 at a sufficient dose to

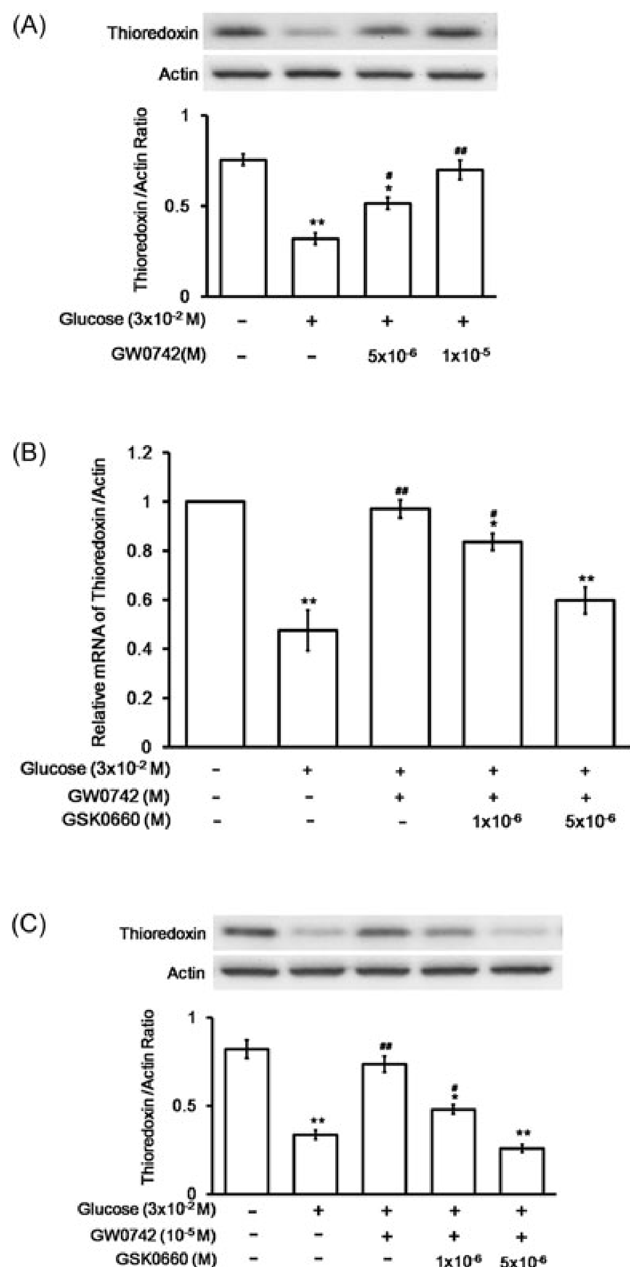

FIGURE 4 GW0742 enhances thioredoxin expression in H9c2 cells. The protein level of thioredoxin was markedly reduced by hyperglycemia and it was dose-dependently reversed by GW0742 (A). In addition, this action of GW0742 was dose-dependently inhibited by GSK0660 at both the mRNA levels of thioredoxin (B) and protein levels of thioredoxin (C). Each column shows mean ± SEM (n = 6). $^{*}P < 0.05$ or $^{**}P < 0.01$ are significantly different from the vehicle-treated control (the first column). $^{\#}P < 0.05$ or $^{\#\#}P < 0.01$ are significantly different from the vehicle-treated under high glucose group (the second column)

inhibit PPARδ as shown in Table 2. Therefore, GW0742 activates PPARδ, which then promotes the expression of Trx2.

3.4 | GW0742 activates PPARδ to lower calcium levels in H9c2 cells

Calcium levels in H9c2 cells were markedly increased by hyperglycemia (Table 1). GW0742 inhibited the intracellular calcium levels in a dose-dependent manner. In addition, this action of GW0742 was dose-dependently reversed by GSK0660 (Table 2). Next, we investigated the possible mechanism(s) for this change.

As shown in Figure 4, GW0742 attenuated the increased calcium levels induced by cardiac contractors, including phenylephrine and potassium chloride. Similar results were not observed in H9c2 cells receiving pretreatment with tiron at the dose effective to inhibit ROS. In addition, cellular calcium levels raised by KBrO3 were also attenuated by GW0742, which was specifically reversed by GSK0660 at a dose sufficient to block PPARδ. However, its reduction by GW0742 was more significant than the effect of tiron, therefore, from the data in Figure 5, it can be concluded that GW0742 activates PPARδ to inhibit free radicals and calcium mobilization resulting in a decrease of calcium levels in H9c2 cells.

4 | DISCUSSION

In the current study, we found that activation of PPARδ may alleviate cardiac hypertrophy in H9c2 cells via antioxidant-like action. Hyperglycemia is used to induce cardiac hypertrophy in cell models and the expression of PPARδ in H9c2 cells has been documented to be significantly reduced under HG conditions.[32] We demonstrated that the PPARδ agonist GW0742 improves cardiac hypertrophy via reduction of free radicals and decreases in intracellular calcium levels. Our results showed that thioredoxin, an antioxidant enzyme, was increased in GW0742-treated H9c2 cells in addition to causing direct inhibition of oxidant $KBrO_3$ induced increases in intracellular calcium levels. This finding is fully consistent with the previous view that PPARδ activation appears to exert most of the direct and indirect inhibitory effects on free radical production in the cardiac system.[29]

GW0742 was developed to activate PPARδ[33] although it has also been found to inhibit free radicals unrelated to PPARδ activation.[25] Therefore, we identified the effects of GW0742 that were sensitive to GSK0660, which is an established antagonist of PPARδ.[34] No effects of GW0742 unrelated to PPARδ activation were observed in the current study.

The embryonic rat heart-derived H9c2 cells[35] showed similar hypertrophic responses to those observed in primary cultured cardiomyocytes.[36] Therefore, we used H9c2 cells to expose to hyperglycemia for induction of a

cardiac hypertrophic model as described in our previous methods.[20] Hyperglycemia was applied with 30 mM glucose in the medium; 35 mM glucose has been reported to produce apoptosis in H9c2 cells.[37] The success of the cell model was confirmed using fluorescence microscopic examination of the H9c2 cell surface area.[20] We found that GW0742 activates PPARδ to improve the cardiac hypertrophy induced by hyperglycemia in H9c2 cells. This finding is consistent with previous views that PPARδ activation inhibits cardiac hypertrophy either induced by phenylephrine[11] or by angiotensin II in vitro.[12] As for the cellular mechanisms for cardiac hypertrophy, it has been established that calcineurin may dephosphorylate NFAT3 and other transcription factors, leading to their nuclear translocation.[38] Then, the nuclear NFAT3 participates in the promotion of hypertrophic gene expression including ANP, BNP, β-MHC, and others to induce cardiac hypertrophy.[3] Similar changes were characterized in H9c2 cells incubated with HG; HG increased cellular calcium levels that could activate calcineurin to work as another potential trigger of the translocation of pro-hypertrophic transcription factors into the nucleus.[39] Moreover, GW0742 activated PPARβ/δ in normal mice and neonatal mouse cardiomyocytes could rapidly induce cardiac angiogenesis through the upregulated expression of calcineurin.[40] However, a decrease of PPARδ expression has been indicated under HG conditions.[32] Therefore, the cellular level of PPARδ expression is varied between diabetic and normal animals. In the current study, GW0742 dose-dependently reversed the expression of PPARδ that was reduced by HG in H9c2 cells showing hypertrophy (Figure 1). In addition, GW0742 also attenuated the calcium levels and hypertrophic signals increased by HG in the same manner. Therefore, the increased mRNA levels of BNP and β-MHC were markedly reversed (Table 1). These effects of GW0742 were totally inhibited by GSK0660, showing the mediation of PPARδ activation.

Cardiac hypertrophy is associated with increased free radicals.[26] Abnormal metabolites induced by diabetes, similar to hyperglycemia in the heart, cause the production of free radicals.[7] Therefore, HG-induced cardiac hypertrophy in H9c2 cells[28] is widely associated with free

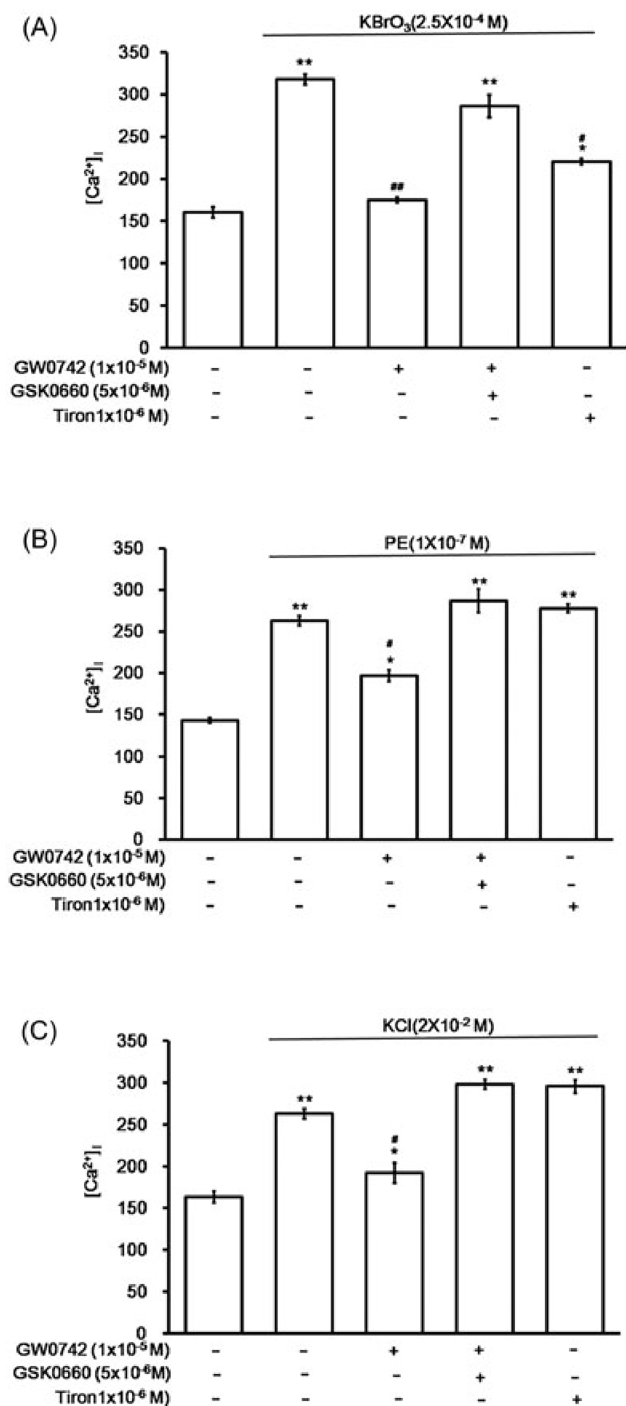

FIGURE 5 GW0742 attenuated intracellular calcium levels ($[Ca^{2+}]_i$) in H9c2 cells. The oxidant, potassium bromated ($KBrO_3$), induced elevation of cellular calcium level that was used as a control (A). In addition, the cardiac contraction enhancers both phenylephrine (PE) and potassium chloride (KCl) were also applied as shown in (B) and (C), respectively. Increased cellular calcium levels were markedly reduced by GW0742 (the second column), which was reversed by GSK0660 (the third column). GSK0660 totally blocked the effects of GW0742 in the elevation of cellular calcium induced by PE and KCl but was only markedly effective in blocking the cellular calcium promoted by $KBrO_3$ as shown in the fourth column. Moreover, antioxidant tiron inhibited the higher cellular calcium induced by KBrO3 but not that by PE or KCl as shown in the fifth column. Each column shows mean ± SEM (n = 6). $^*P < 0.05$ or $^{**}P < 0.01$ are significantly different from the vehicle-treated control (the first column). $^{\#}P < 0.05$ or $^{\#\#}P < 0.01$ are significantly different from the stimulant-treated group (the second column)

radicals. We also confirmed this view in the current study using fluorescence microscopic examination because the superoxide and ROS levels were both increased in H9c2 cells incubated with HG. In addition, GW0742 inhibited changes in superoxide and/or ROS levels, and this inhibition was totally reversed by GSK0660. Therefore, GW0742 inhibited free radicals through PPARδ activation in H9c2 cells.

PPARδ positively regulates antioxidant genes to eliminate excessive ROS. PPARδ is known to be involved in transcription through direct binding of the PPAR/the retinoid X receptor (RXR) heterodimer to transcriptional factors.[41] In a review article,[29] the authors thought that PPARδ activation may exert transcriptional regulation of the expression of several endogenous antioxidants, including Mn-superoxide dismutase (SOD), Cu, Zn-SOD, catalase, and glutathione peroxidase. In addition, PPARδ also regulates angiotensin II-induced stimulation to reduce ROS generation in cardiovascular systems.[42] When oxidative stress increased, PPARδ could directly regulate coactivator 1 (peroxisome proliferator-activated receptor-γ coactivator-1α [PGC-1α] and β) to maintain mitochondrial biogenesis and regulate the myocardial lipid and glucose metabolism.[43] We also found that GW0742 can promote the expression of Trx2, a mitochondrial antioxidant, which was diminished in H9c2 cells by hyperglycemia.[31] This action of GW0742 was blocked by GSK0660 dose-dependently. Trx2 can decrease ROS induced by HG, as described previously.[44] Therefore, the increase of endogenous enzymes showing antioxidant-like action by PPARδ activation seems important in the reduction of free radicals induced by GW0742 in H9c2 cells. This finding could be considered to be an indirect effect of PPARδ activation on the production of free radicals in H9c2 cells.

Cardiac hypertrophy induced contractile abnormalities are accompanied by alterations in cellular calcium transient levels.[45] Free radicals are known to increase cellular calcium levels[46] that are linked to promoting the calcineurin-NFAT signaling pathway for cardiac hypertrophy.[47] In the current study, prolonged exposure to hyperglycemia resulted in a significant increase in basal levels of Ca^{2+}, which have been linked to hypertrophy through the generated free radicals in H9c2 cells.[20] Similarly, aspirin decreases the calcium level elevated by angiotensin-II to improve hypertrophy in cardiomyocytes from mice.[48] One possible reason was the increase of L-type Ca^{2+} channel density and the number of dihydropyridine binding sites in cardiac myocytes as hypertrophy develops.[49]

PPARδ may modulate calcium homeostasis by directly or indirectly regulating oxidative stress induced by free radicals.[29] First, we applied potassium bromate ($KBrO_3$) as an oxidizing agent to induce an increase of calcium levels in H9c2 cells.[50] In H9c2 cells, the increase of the calcium levels induced by $KBrO_3$ was attenuated by PPARδ activation. This effect was also markedly inhibited by tiron, indicating that PPARδ may modulate calcium homeostasis through regulation of oxidative stress. Moreover, GW0742 also attenuated the increase in calcium levels induced by phenylephrine and potassium chloride, which were not eliminated by the antioxidant tiron. Therefore, PPARδ can directly regulate calcium mobilization independently of the changes in oxidative stress induced by $KBrO_3$. This is consistent with another PPARδ activator, L-165041, that can suppress calcium levels elevated by angiotensin-II in H9c2 cells.[51] Therefore, the obtained result for GW0742 is a PPARδ-dependent but nongenomic effect, which does not require the involvement of endogenous enzymes. Furthermore, the effect of GW0742 on the calcium level was more significant than tiron, indicating both mechanisms are involved in the effect of PPARδ activation. Taken together, inhibition of free radicals in addition to the direct blockade of calcium mobilization induced by GW0742 via PPARδ activation can be identified.

This report had several limitations. A diabetic animal model was not used in the current study. In addition, the interactions of GW0742 involved in myocardial hypertrophy and cardiac contractility in vivo remained unclear. Further investigations are required to evaluate the pharmacokinetics and pharmacodynamics of GW0742 in the future.

In conclusion, our findings suggest that GW0742 can activate PPARδ to attenuate the cellular calcium level via a direct blockade and indirectly through the reduction of free radicals, which was also produced by a direct inhibition in addition to the indirect mechanism via an activation of endogenous antioxidant enzymes, leading to alleviation of cardiac hypertrophy induced by hyperglycemia in H9c2 cells. Therefore, we identified the potential mechanisms of GW0742 through PPARδ activation for the alleviation of cardiac hypertrophy in H9c2 cells.

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