Expression of Glucocorticoid Induced Leucine Zipper (GILZ) in Cardiomyocytes

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Abstract

Glucocorticoids (GCs) are frequently prescribed pharmacological agents most notably for their immunosuppressive effects. Endogenous GCs mediate biological processes such as energy metabolism and tissue development. At the cellular level, GCs bind to the Glucocorticoid Receptor (GR), a cytosolic protein that translocates to the nuclei and functions to alter transcription upon ligand binding. Amongst a long list of genes activated by GCs is the Glucocorticoid Induced Leucine Zipper (GILZ). GC induced GILZ expression has been well established in lymphocytes and mediates GC induced apoptosis. Unlike lymphocytes, cardiomyocytes respond to GCs by gaining resistance against apoptosis. We determined GILZ expression in cardiomyocytes *in vivo* and *in vitro*. Expression of GILZ in mouse hearts as a result of GC administration was confirmed by Western blot analyses. GCs induced dose and time dependent elevation of GILZ expression in primary cultured rat cardiomyocytes, with dexamethasone (Dex) as low as 0.1 M being effective. Time course analysis indicated that GILZ protein levels increased at 8 hr and peaked at 48 hr after exposure to 1 M Dex. H9c2(2-1) cell line showed a similar response of GILZ induction by Dex as primary cultured rat cardiomyocytes, providing a convenient model for studying the biological significance of GILZ expression. With corticosterone (CT), an endogenous form of corticosteroids in rodents, 0.1–2.5 M was found to induce GILZ in H9c2(2-1) cells. Time course analysis with 1 M CT indicated induction of GILZ at 6 hr with peak expression at 18 hr. Inhibition of the GR by mifepristone led to blunting of GILZ induction by GCs. Our data demonstrate GILZ induction in cardiomyocytes both *in vivo* and *in vitro by GCs*, pointing to H9c2(2-1) cells as a valid model for studying the biological function of GILZ in cardiomyocytes.

Keywords

Glucocorticoid Induced Leucine Zipper; Glucocorticoids; H9c2(2-1) cells; primary neonatal cardiomyocytes

Introduction

GCs are among the most commonly prescribed pharmaceutical agents. Therapeutic actions of GCs are largely attributed to their anti-inflammatory and immunosuppressive properties.

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At the cellular level, GCs are best known for inducing apoptosis in lymphocytes and the molecular mechanism of such action has been extensively studied. GILZ was originally discovered when characterizing genes induced by Dex using a thymus subtraction library (1). Since then, GILZ has been reported to mediate apoptosis of lymphocytes, T-helper cell differentiation, function of dendritic cells, increasing Na^+ transport in the kidney and inhibition of Ras driven tumorigenesis (1–5).

Given the nature of being a leucine zipper, GILZ was originally thought to function as a transcription factor. Lack of a canonical DNA binding domain in GILZ has ruled out direct transcriptional regulation. Amino acid analysis of GILZ revealed a central leucine zipper domain (LZ; 76–97 aa) with the ability to dimerize with other leucine zipper proteins (6, 7). The N-terminal domain (N-ter; 1–75 aa) of GILZ has been shown to bind and inhibit proto-oncogene RAF, a serine/threonine-kinase (8). The Tuberous Sclerosis Complex box (TSC box; 61–75 aa) located in the N-terminal domain of GILZ has been reported to bind Ras (9). GILZ also contains a C-terminal proline and glutamic acid rich region that has been reported to interact with and inhibit NF-B. Therefore GILZ may regulate transcription through interaction with signaling molecules and transcription factors.

GILZ is expressed at a high basal level in the lung, brain and skeletal muscle (10). The gene encoding GILZ contains multiple Glucocorticoid Response Elements (GRE) in the promoter, and putative binding sites for a number of transcription factors, including STAT6, Nuclear factor of Activated T-Cells (NFAT), Oct-1, c-myc, Forkhead, cyclic AMP Response Element-Binding protein (CREB), and Estrogen Receptor (11, 12). To date, there is no literature describing the regulation and function of GILZ gene in the heart.

In cardiomyocytes, GCs have been found to be cytoprotective and prevent several damaging agents from inducing apoptosis (13). Identifying genes induced by GCs in cardiomyocytes allows the understanding of underlying mechanism of GC induced cytoprotection. Affymetrix microarray experiments revealed that CT caused the up-regulation of 140 genes and down-regulation of 108 in cardiomyocytes. As expected, GILZ expression was found to be induced in primary cultured rat cardiomyocytes by CT (13).

H9c2(2-1) cells were derived from embryonic heart tissue of BD1X rat and express biomarkers of cardiac muscle, such as rapidly activating cardiac L-type Ca^{2+} channel and Small Nuclear Ribonucleoprotein-associated Protein N (SmN), a cardiac specific splicing protein (14). This cell line has been widely used as an *in vitro* model for studying molecular events of cardiomyocytes (15, 16). Unlike primary cultured cardiomyocytes, H9c2(2-1) can be subcultured and maintained under tissue culture condition, providing the convenience for studying the regulation and function of GILZ gene. In this study, we compared GILZ expression between primary cultured cardiomyocytes and H9c2(2-1) cells, and address whether H9c2(2-1) cells can serve as an experimental model for studying the regulation and function of GILZ in cardiomyocytes.

Materials and Methods

1. Chemicals

All chemicals were obtained from Sigma-Aldrich (St. Louis, MO) unless otherwise indicated. Pharmacological inhibitors were obtained from Enzo Life Sciences (Plymouth Meeting, PA).

2. *In Vivo* Study

Male C57BL6 mice at 8 weeks old were administered 20 mg/kg Dex. At 24 hrs after, the hearts were removed and immediately frozen in liquid nitrogen. Proteins were extracted by grinding tissues with a mortar and pestle in a lysis buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM Sodium Pyrophosphate, 1 mM beta-Glycerophosphate, 1 mM Na_3VO_4, 1 g/mL Leupeptin, from Cell Signaling, Boston, MA). After lysis in a 4°C orbital shaker for 1 hr, protein concentration was measured by the Bradford method (Bio-Rad, Hercules CA) and Western blot analysis was performed to determine GILZ protein levels.

3. Cell Culture and Treatment of Drugs

Primary cultured cardiomyocytes were prepared from 1 to 2 days-old neonatal Sprague-Dawley rats as described (17). These myocytes were seeded at a density of 2×10^6 cells per 100 mm dish or 0.3×10^5 cells per well in 6-well plates in low glucose Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% Penicillin/Streptomycin. Three days after plating, cells were treated with Dex.

H9c2(2-1) cells, obtained from ATCC, were seeded in 6-well plates at 3×10^5 cells per well and grown to 80% confluency. The cells were starved in serum free media 16 hr before treatment with corticosterone (CT), estrogen, cholesterol, testosterone or L-Thyroxine at a final concentration of 1 M for 8 hrs. Inhibitors were administered 1 hr before 1 M CT treatment. Final concentrations of inhibitors were as follows: PD-98059 20 M, Wortmannin 100 nM, LY 294002 10 M, H-89 10 M, mifepristone 1 M, SB-202190 10 M, FR 180204 (Santa Cruz Biotechnology Inc, CA) 10 M and Rapamycin 5 ng/ml. Cells were harvested in the lysis buffer (see Section 2) for measurements of GILZ protein by Western blot analyses.

4. Real-Time PCR (qPCR)

Total RNA was extracted with TRIzol and converted to cDNA using cDNA synthesis kit (Fermentas) with random hexamers as primers. For PCR, specific primers against rat GILZ and GAPDH were purchased from the Integrated DNA Technologies (IDT, San Diego CA) with the sequence of 5′-AGC TGA ACA ACA TAA TGC GCC AGG-3′ or 5′- ATC TTG TTG TCT AGG GCC ACC ACA-3′ as forward or reverse primer for GILZ, and 5′-CCT CTC TCT TGC TCT CAG TAT-3′ or 5′-GTA TCC GTT GTG GAT CTG ACA-3′ as forward or reverse primer for GAPDH. Real time PCR was performed on the CFX96 Thermal Cycler (Bio-Rad) using Syber Green Fermentas) for product detection after 10 mins of denaturation at 95°C followed by 35 cycles of 95°C for 15 sec, 60°C for 30 sec and 72°C for 30 sec. At the end of PCR, melting curve analysis was carried out to verify product specificity. CFX Manager™ software (BioRad, Hercules, California) installed in CFX96

Thermal Cycler was used for quantification of Relative Quantity [C_t] as indicated in the manual, using an equation of $E^{[C_{t(min)}-C_{t(sample)}]}$, where E=efficiency of primer and probe set, $C_{t(min)}$ is average $C_{(t)}$ for the sample with the lowest average C_t, and $C_{t(sample)}$ is average C_t for the Sample.

5. Western Blot

Proteins were separated by SDS-polyacrylamide (12%) gel electrophoresis and transferred to a PVDF membrane at 60V for 3 hr (Bio-Rad). Membranes were blocked with 10% milk in TBS-tween solution for 2 hours, followed by incubation with antibodies against rabbit or mouse GILZ (Santa Cruz Biotechnology, CA) at 1:200 dilution. Vinculin, serving as a loading control, was recognized by a monoclonal antibody at 1:1000 dilution. The bound antibodies were detected using an Enhanced Chemiluminescence reaction after blotting with the secondary antibodies conjugated with Horseradish Peroxidase.

6. Image Quantification and Statistical Analysis

NIH Image J software was utilized to quantify the intensity of bands from Western blot analyses. The density of GILZ band was normalized to that of vinculin within the same sample with the value of the control sample being set as 1. Student's t-test or ANOVA followed by Bonferroni correction was used to determine significant differences (P<0.05) between groups. Significant difference is indicated as * by comparing to the corresponding control.

Results

Dexamethasone Induces GILZ in Mouse Myocardium

To address whether GCs induce GILZ in the myocardium, C57BL6 mice were administered with 20 mg/kg Dex (i.p.) with an equal volume of vegetable oil as a vehicle control. This dose has been shown previously from our laboratory to protect the heart from myocardial injury (18). The hearts were collected at 24 hr after for measurements of GILZ protein by Western blot. The results indicated that mice dosed with Dex showed elevated levels of GILZ protein compared to the control group (Figure 1).

Dexamethasone Induces GILZ in Neonatal Rat Cardiomyocytes and H9c2(2-1) Cells

Primary cultured rat neonatal cardiomyocytes are commonly used *in vitro* model for studying cellular and molecular events induced by pharmacological agents. To characterize GILZ expression, we performed dose response and time course studies. Within 8 hrs of Dex treatment at various doses, 0.1 M Dex treatment caused significant induction of GILZ protein while 1.0 M Dex caused the highest level of GILZ protein induction (Figure 2A). Time course analysis of GILZ expression was performed using 1 M Dex. The results indicate the peak expression of GILZ protein expression at 48 hours with Dex treatment in primary neonatal cardiomyocytes (Figure 3A).

With H9c2(2-1) cells, a dose response similar to primary cultured cardiomyocytes was observed, with GILZ being induced at 0.1 M Dex and 0.5 M Dex caused the highest level of GILZ protein induction (Fig 2B). The time course study showed GILZ induction at 8 hrs,

similar to primary cultured cardiomyocytes, but peak GILZ induction at 24 hr (Fig 3B). Although minor differences exist between the two cell models in dose response and time course of GILZ induction by GCs, H9c2(2-1) cells can substitute primary cultured cardiomyocytes for initial study of regulatory mechanism of GILZ expression and the function of GILZ protein.

Corticosterone Induces GILZ in H9c2(2-1) Cells

CT is the form of endogenous GCs in rodents and has been used to study the protective effect against apoptosis in cardiomyocytes (13). The dose response experiments were performed over an 8 hr period with 0.25 to 2.5 M CT. The highest dose used, 2.5 M, caused a maximal GILZ induction (Figure 4A). Time course analysis was performed using 1 M CT and results indicated GILZ expression peaked at 18 hr (Figure 5A). qRT-PCR was performed to measure GILZ mRNA levels in cells responding to CT. GILZ transcript elevation was consistent with protein induction in both dose response and time course (Figures 4B, 5B).

GILZ Expression Is Glucocorticoid Receptor Dependent

GILZ gene contains multiple GREs in the promoter, suggesting a role of GR in GILZ expression. To test whether GILZ induction by GCs is mediated through GR, we pre-treated primary neonatal cardiomyocytes or H9c2(2-1) cells with mifepristone (MF), a GC receptor antagonist, prior to exposing cells to 1M Dex or CT for 8 hr. MF blocked the induction of GILZ in primary neonatal cardiomyocytes and H9c2(2-1) cells (Fig. 6A, 6B), suggesting that GC induced GILZ expression is indeed dependent on the GR.

Previous work from our laboratory and others indicate that GCs can activate phosphatidylinositol 3-kinase (PI3 kinase) (19–21). Whether GCs activate or inhibit MAP kinases is controversial, since both activation and inhibition have been reported (22–26). To address whether these signaling pathways are involved in the induction of GILZ by GCs, pharmacological inhibitors were utilized. Analyses of GILZ mRNA or protein levels suggest that GILZ is primarily regulated by the GR (Figure 7A, 7B). The PI3 Kinase inhibitor, wortmannin as well as the mTOR inhibitor rapamycin failed to inhibit GILZ expression. Interestingly, H89, a PKA inhibitor, as well as the p38 inhibitor SB202190 induced GILZ expression when given alone and enhanced GILZ induction by CT. These findings suggest that GILZ expression is coupled to GR activation by GCs and that PI3K pathway does not play a role in GILZ induction.

Progesterone Induces GILZ in H9c2(2-1) Cells

GILZ has been reported induced by steroid hormones other than glucocorticoids in selective tissues (5, 11). To determine if steroids other than GCs induce GILZ in cardiomyocytes, we treated H9c2(2-1) cells with 1 M cholesterol, estrogen, thyroxine, progesterone, retinoic acid or testosterone with corticosterone as a positive control. Estrogen, testosterone, thyroxine, retinoic acid, or cholesterol did not cause GILZ elevation (Fig 8). Progesterone increased GILZ expression in H9c2(2-1) cells (Fig 8).

Discussion

In this report, we describe the ability of GCs to induce GILZ in the myocardium, primary cultured cardiomyocytes and H9c2(2-1) cell line. GILZ induction was dose and time dependent in both H9c2(2-1) cells as well as primary neonatal cardiomyocytes. The hormone receptor antagonist, mifeprestone, blocks GILZ expression in both primary cardiomyocytes and H9c2(2-1) cells, suggesting GILZ expression is dependent on GR activation. In addition to GCs, progesterone was found to induce GILZ expression in H9c2(2-1) cells.

The identification and characterization of GILZ in T-lymphocytes have suggested that GILZ participates in signaling events regulating cell death or survival (1). Given our previous reports showing that GCs contribute to cardiomyocyte cytoprotection (13), a valid cardiomyocyte cell model is necessary to study the role of GILZ in survival elicited by GCs. Cardiomyocyte cell lines provide a time and labor efficient model with reproducibility for investigating molecular mechanisms underlying pathologies associated with heart diseases. Established cardiomyocyte cell lines include HL-1 and H9c2(2-1) (27). The HL-1 cell line was derived from mouse atrial tumors and retains contractility but is difficult to maintain in culture. In contrast, H9c2(2-1) cells were derived from the embryonic rat myocardium. Although H9c2(2-1) cells do not contract, they retain many molecular and biochemical characteristics of primary cardiomyocytes, such as membrane morphology, G-protein signaling and electrophysiological properties (28, 29). Like primary cultured neonatal cardiomyocytes, H9c2(2-1) cells exhibit similar characteristics during hypertrophy (30, 31), suggesting that H9c2(2-1) cells serve as a valid model of cardiomyocytes.

We show a similar pattern of Dex induced GILZ in primary cultured cardiomyocytes and H9c2(2-1) cells. Peak levels of GILZ expression were observed at a slightly lower concentration in H9c2(2-1) cells and began to show saturation or less effective above 0.5 M Dex (Fig 2B). Primary cells showed a peak level of GILZ at 1.0 M Dex, which was maintained at higher concentrations of Dex (Fig 2A,B). Both H9c2(2-1) cells and primary neonatal cardiomyocytes had sustained GILZ elevation over an extended period of time. However primary cells were able to maintain a high level of expression after 48 hr (Fig 3A, 3B). These suggest that primary cultured cardiomyocytes may contain more abundant GR than H9c2(2-1) cells.

CT, the primary GC in rodents, induced GILZ in a time and dose dependent manner in H9c2(2-1) cells (Fig 4,5). Although peak levels of expression were observed at higher concentrations as compared to Dex induction (1.0 M Dex vs. 2.5 M CT), 1.0 M GC is a good dose for inducing GILZ in both cell systems. Furthermore, GILZ expression is detected at earlier time points and is maintained for a longer time in the presence of Dex as compared to CT. This profile fits with the fact that Dex, a synthetic GC, has a longer half life than CT. Our study is consistent with differences between GC and Dex stimulation as reported in other cell systems (32).

GCs induce transcription of target genes through activating GR. The mechanism involves GR homodimerization or heterodimerization with other transcription factors followed by

DNA binding to induce or repress genes downstream of GC signaling. In addition to this well established genomic effect, GCs also elicit non-genomic effects through protein-protein interactions. In this manner, GR can activate or suppress gene expression independent of DNA binding. For instance, GCs can activate kinase-mediated signaling pathways within minutes through nongenomic GR mediated signaling to regulate gene expression (20, 33, 34). Since induction of GILZ requires hours after Dex or CT exposure, we believe that the genomic effect of GCs is critical for GILZ expression.

Signaling pathways influenced by GCs were screened to determine the mechanism of GILZ induction. Among the inhibitors used, only the GR antagonist Mifepristone attenuated GILZ expression, indicating that GR is responsible for GILZ induction (Fig 7). This is logical because the gene encoding GILZ contains multiple Glucocorticoid Response Elements (GRE) in the promoter (11, 12). Interestingly, p38 inhibitor as well as pKA inhibitor alone induced the expression of GILZ. These findings suggest that cAMP-PKA and p38 MAPK signaling pathways serve to negatively regulate GILZ expression. Both p38 and PKA inhibitors induce GILZ protein independent of increases in mRNA (FIG 7B). Both pathways have also been reported to mediate protein stability in cardiomyocytes. For example, p38 has been reported to directly phosphorylate p300 in cardiomyocytes, which lead to the subsequent degradation of p300 by the proteasome (35). Furthermore, PKA activity has been reported to induce proteasome activation in cardiomyocytes (36, 37). It is therefore reasonable to predict both PKA and p38 pathways regulate GILZ at the translational or posttranslational level in cardiomyocytes.

In addition to GCs, progesterone was also observed to increase GILZ expression. Progesterone receptor (PR) is expressed in mammalian myocardium (38–42). PR belongs to the same family of nuclear receptor as GR, and binds to a common Hormone Response Element (HRE) in the promoter of targeted genes. Progesterone has been reported to induce transcription of genes containing HREs (43–46). HREs are often referred to as GREs when glucocorticoids are the activating factor. At an equivalent dose of 1 M, progesterone is less potent than CT in inducing GILZ protein levels (Fig 8). This suggests that GILZ expression is more specifically coupled to GR binding to its cognate HRE. Additionally GRE has been reported to be promiscuous and retain the ability to be activated by PR (47, 48). High concentrations of PG have been reported to induce GRE activity (49). Nevertheless, GILZ induction correlates with cytoprotection, since both GCs and PG exhibit cytoprotective effect in cardiomyocytes (13, 49, 50). Further studies will examine whether GILZ indeed functions as a cytoprotective gene in cardiomyocytes.

Acknowledgments

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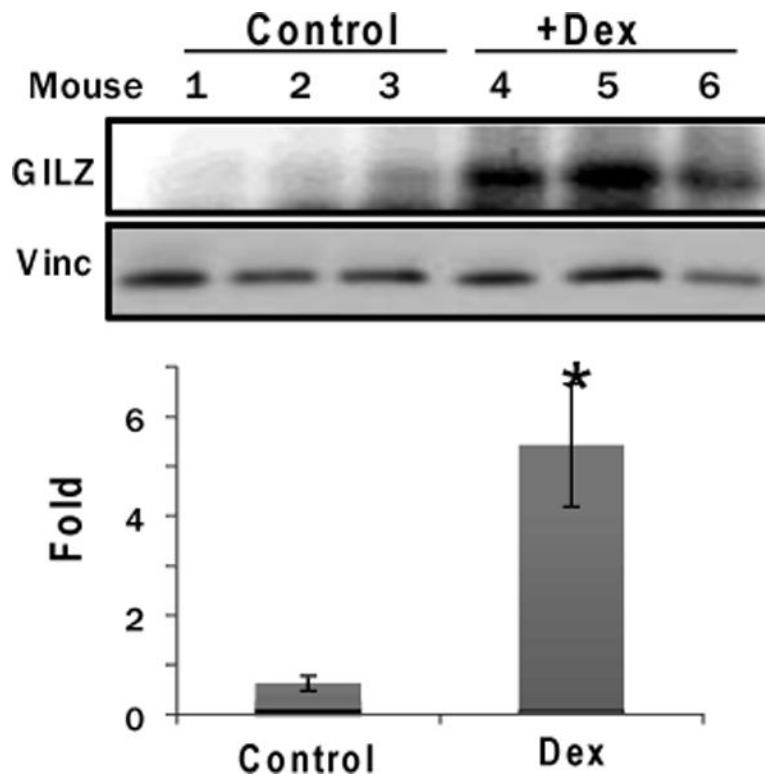
Figure 1. Dex Induces GILZ in the Mouse Myocardium
Male C57BL6 mice (8 weeks old) were treated (i.p.) with 20 mg/kg Dex or vegetable oil as a vehicle control for 24 hours. The myocardial tissues were harvested for Western blot analyses to detect GILZ with vinculin (vinc) as a loading control. The band intensities were quantified using NIH Image J software and were presented as average ± standard deviation from 3 animals, with the number of control being set as 1. Significant difference (p<0.05) between Dex treated versus vehicle control is indicated by *.
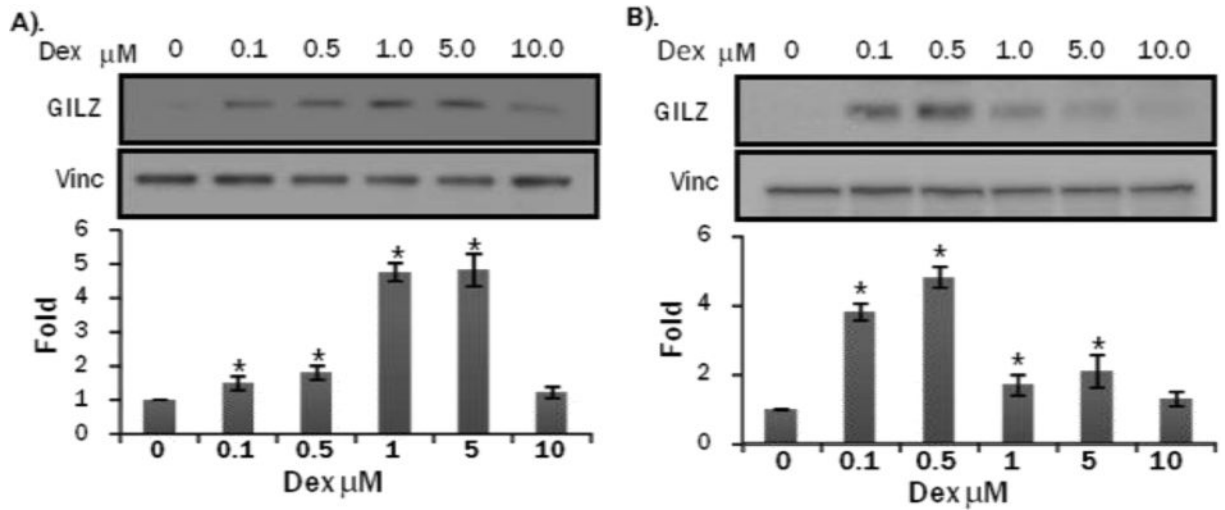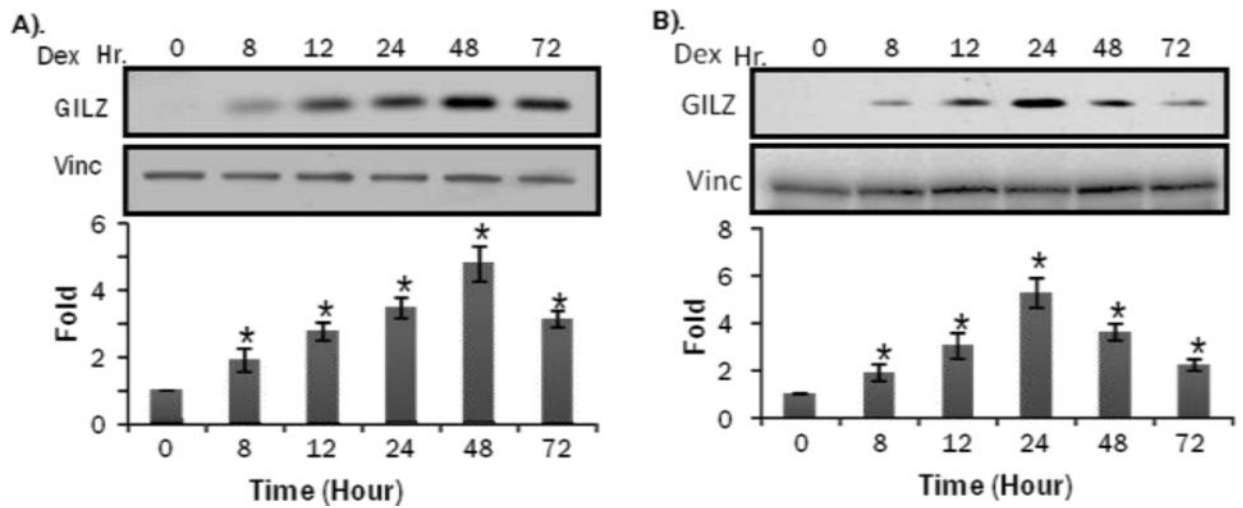
Figure 2. Dex Dose Dependent GILZ Induction in Cultured Cardiomyocytes
Primary cultured neonatal rat cardiomyocytes (**A**) or H9c2(2-1) cells (**B**) were treated with Dex in indicated doses for 24 hrs. Cells were harvested for Western blot to detect GILZ with vinculin (vinc) as a loading control. The intensities of the bands were quantified using NIH Image J software. The quantitative data represent average ± standard deviations from three independent experiments. ANOVA followed by Bonferroni correction was used for comparison of treated group with control using data from three independent experiments and the significance of difference is indicated by (*).

Figure 3. Time Dependent GILZ Induction by Dex in Cultured Cardiomyocytes
Primary cultured neonatal rat cardiomyocytes (**A**) or H9c2(2-1) cells (**B**) were treated with 1 M Dex for indicated time. Cells were harvested for Western blot to measure GILZ protein levels with vinculin (vinc) as loading control. The intensities of the bands were quantified and significant difference from 0 hr, as indicated by * (p<0.05), was determined as described in Figure 2.
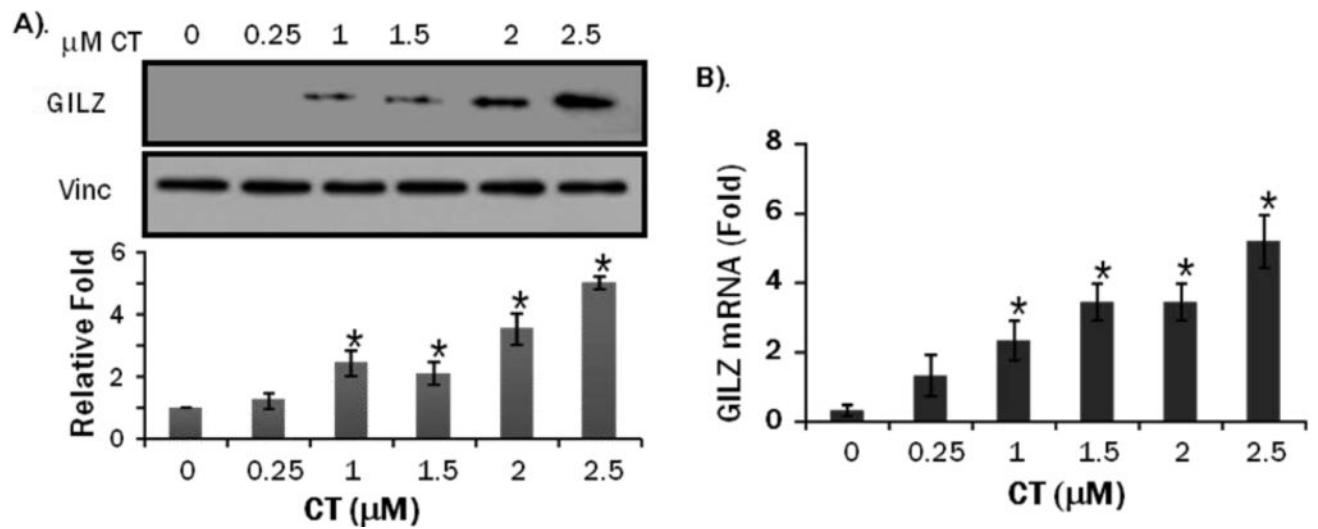
Figure 4. CT Induces GILZ Protein and mRNA in a Dose Dependent Manner in H9c2(2-1) Cells
H9c2(2-1) cells were treated with CT at indicated doses for 8 hrs. Proteins or total RNAs were harvested from these cells for Western blot analyses (**A**) or real time RT-PCR (**B**). The intensities of the bands were quantified and significant difference from control (0 M), as indicated by * ($p < 0.05$), was determined as described in Figure 2.
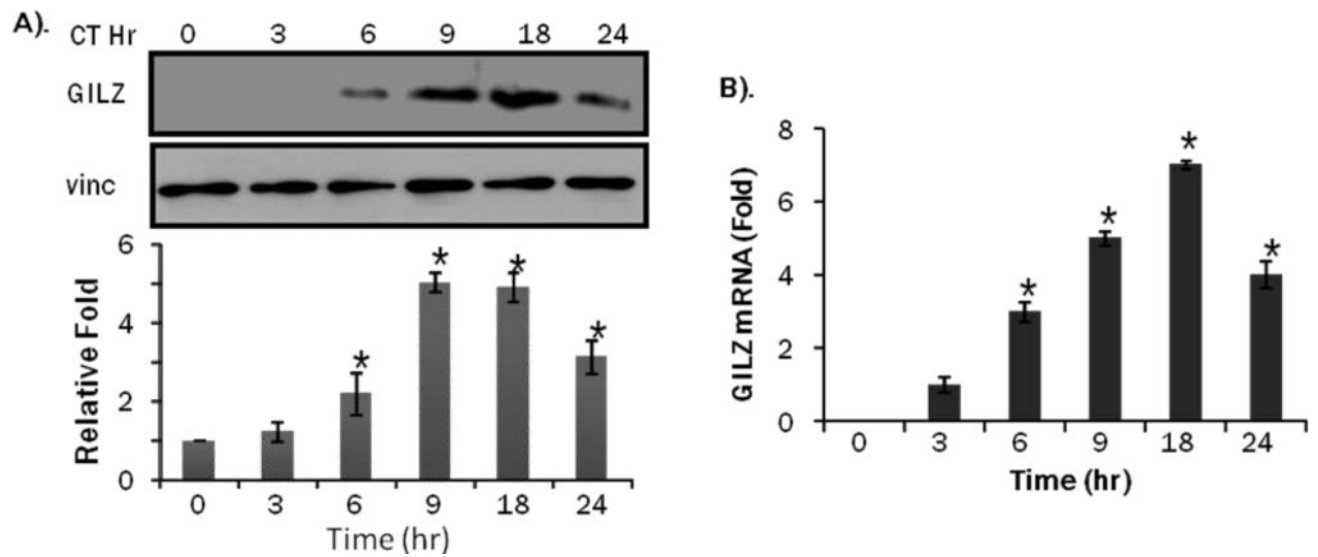
Figure 5. CT Induces GILZ Protein and mRNA in a Time Dependent Manner in H9c2(2-1) Cells
H9c2(2-1) cells were treated with 1 M CT for indicated time. Proteins or total RNAs were harvested from these cells for Western blot analyses (**A**) or real time RT-PCR (**B**). The intensities of the bands were quantified and significant difference from 0 hr control, as indicated by * ($p < 0.05$), was determined as described in Figure 2.
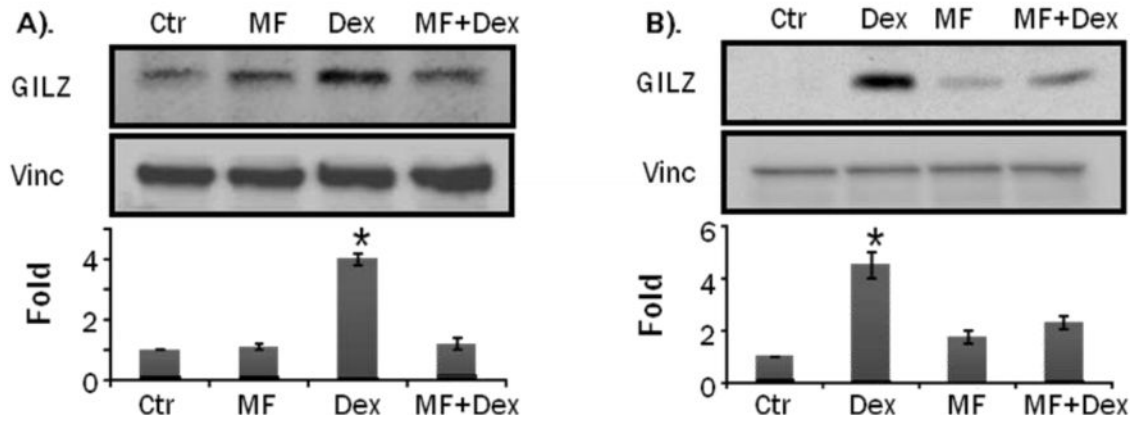
Figure 6. Mifepristone Blocks GILZ Induction by Dex

Primary cultured neonatal rat cardiomyocytes (**A**) or H9c2(2-1) cells (**B**) were treated with 1 M mifepristone (MF) 1 hr prior to Dex treatment for 24 hrs. Cells were harvested for Western blot to measure GILZ with vinculin (vinc) as loading control. The intensities of the bands were quantified and significant difference from control, as indicated by * ($p<0.05$), was determined as described in Figure 2.
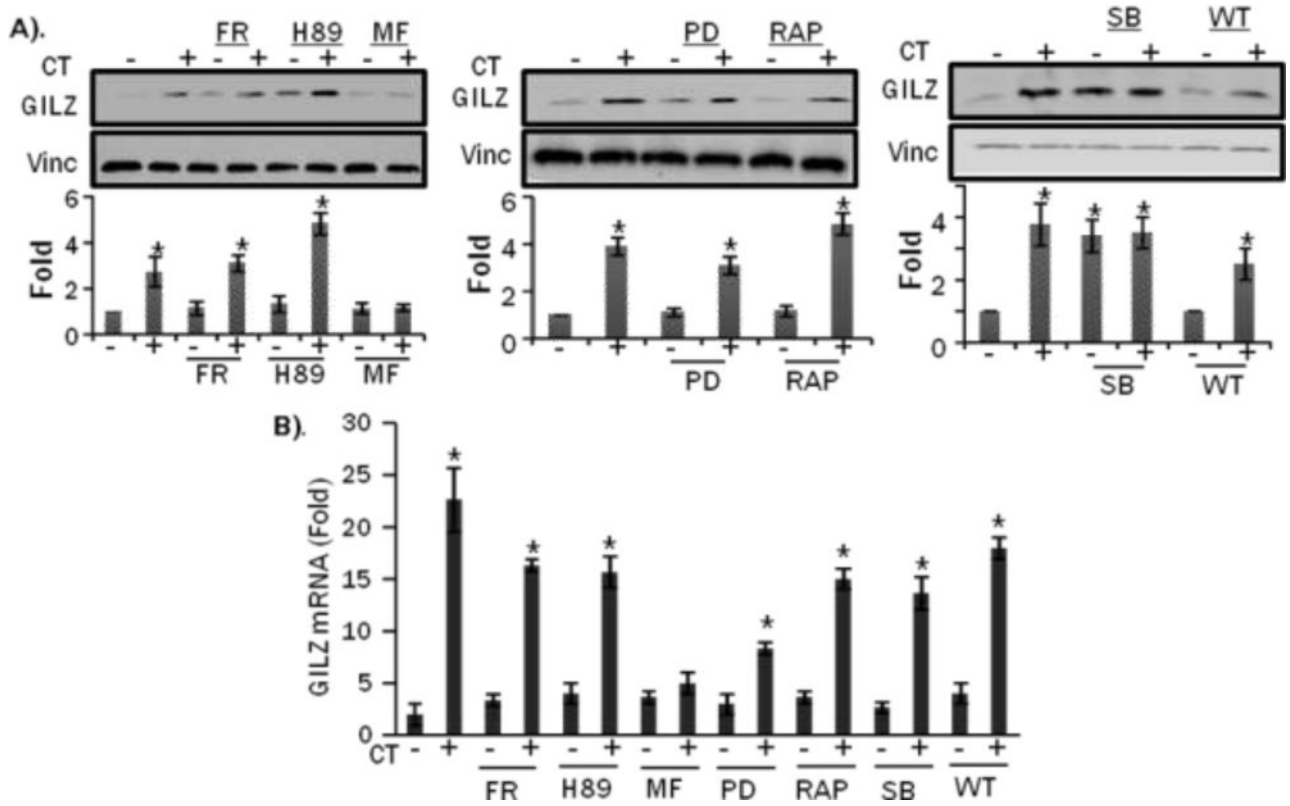
Figure 7. Effect of Pharmacological Inhibitors on GILZ Induction by CT
H9c2(2-1) cells were treated with ERK inhibitor (FR180204, 10 M), PKA inhibitor (H89, 10 M), GR inhibitor Mifepristone (MF, 1 M), MEK inhibitor (PD98059, 10 M), mTOR inhibitor Rapamycin(RAP, 5 ng/mL), p38 inhibitor (SB202190, 10 M), or PI3 Kinase inhibitor Wortmaninn (WT, 100 nm) for 1 hr before 1 M CT treatment for 8 hrs. Proteins or total RNA were harvested from these cells for Western blot (**A**), or real time RT-PCR (**B**). The intensities of the bands were quantified and significant difference between CT plus drug treated from drug control, as indicated by * (p<0.05), was determined as described in Figure 2.
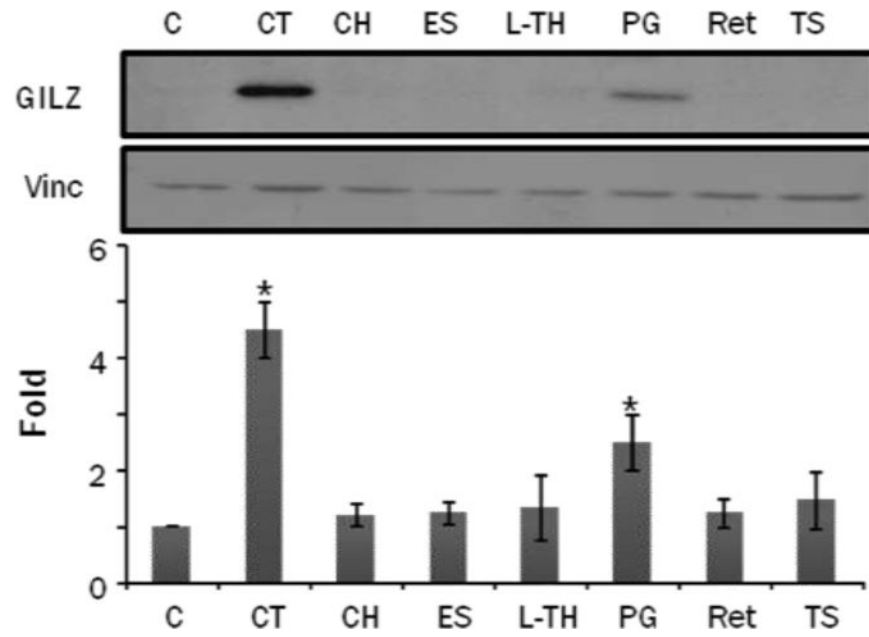
Figure 8. Effect of Nuclear Receptor Agonists in GILZ Induction
H9c2(2-1) cells were treated with 1 M corticosterone (CT), cholesterol (CH), estrogen (ES), L-Thyroxen (L-TH), progesterone (PG), retinoic acid (Ret) or testosterone (TS) for 8 hrs. Cells were harvested for Western blot to measure GILZ protein levels with vinculin (Vinc) as a loading control. The intensities of the bands were quantified and significant difference from control, as indicated by * ($p < 0.05$), was determined as described in Figure 2.