Excess of glucocorticoid induces myocardial remodeling and alteration of calcium signaling in cardiomyocytes

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Abstract

Ventricular dysfunction is one of the important side effects of the anti-inflammatory agent, glucocorticoid (GC). The present study was undertaken to examine whether abnormal calcium signaling is responsible for cardiac dysfunction due to an excess of GC hormone. The synthetic GC drug, dexamethasone (DEX), significantly ($P<0{\cdot}001$, $n=20$) increased heart weight to body weight ratio, left ventricular remodeling, and fibrosis. The microarray analysis showed altered expression of several genes encoding calcium cycling/ion channel proteins in DEX-treated rat heart. The altered expression of some of the genes was validated by real-time PCR and western blotting analyses. The expression of the L-type calcium channels and calsequestrin was increased, whereas sarcoendoplasmic reticulum calcium transport ATPase 2a (SERCA2a) and junctin mRNAs were significantly reduced in DEX-treated rat left ventricular tissues. In neonatal rat ventricular cardiomyocytes, DEX also increased the level of mRNAs of atrial- and brain natriuretic peptides, L-type calcium channels, and calsequestrin after 24 h of treatment, which were mostly restored by mifepristone. The caffeine-induced calcium release was prolonged by DEX compared to the sharp release in control cardiomyocytes. Taken together, these data show that impaired calcium kinetics may be responsible for cardiac malfunction by DEX. The results are important in understanding the pathophysiology of the heart in patients treated with excess GC.

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Introduction

Glucocorticoids (GCs) are among the most frequently used drugs prescribed for the treatment of various disorders such as inflammatory disease, asthma, chronic obstructive pulmonary disease, and autoimmune diseases (Iuchi *et al.* 2003). However, chronic use of GCs causes several adverse side effects such as hyperglycemia, obesity, and cardiovascular complications including hypertension, atherosclerosis, and heart failure (Grunfeld & Eloy 1987, Saruta 1996). Hypertension for a prolonged duration may cause considerable changes in myocardium such as tissue remodeling, ventricular hypertrophy, and myocyte dysfunction ultimately leading to heart failure (Maron 2002, Xu *et al.* 2007). Initially, cardiac hypertrophy is beneficial compensating the increased workload on the heart; however, in the long run, it becomes maladaptive and decompensatory resulting in cardiac dysfunction (Feldman *et al.* 1993). It is well known that hypertrophic growth of the myocytes is accompanied by increased collagen synthesis leading to the development of myocardial fibrosis. Myocardial fibrosis leads to increased stiffness of the myocytes, which is primarily responsible for contractile abnormality and cardiac dysfunction (Weber & Brilla 1991).

At the cellular level, the contractile power during excitation–contraction coupling (E–C coupling) is governed by a mechanism known as Ca^{2+}-induced Ca^{2+} release (CICR) (Xu *et al.* 2007). CICR is a process in which, upon myocyte membrane depolarization, Ca^{2+} influx, through cell-surface L-type Ca^{2+} channels mainly and reverse-mode Na^{+}/Ca^{2+} exchanger to a lesser extent (Sipido *et al.* 1997, Maier & Bers 2002), activates ryanodine receptor (RyR) calcium release from the sarcoplasmic reticulum (He *et al.* 1997). The release of Ca^{2+} from the RyR clusters is visualized as Ca^{2+} sparks, and these Ca^{2+} sparks amplify the initial Ca^{2+} signal and combine to produce an elevation of cell-wide myoplasmic $[Ca^{2+}]_i$ called Ca^{2+} transient. This increase in cytosolic Ca^{2+} concentration leads to the activation of the contractile proteins and hence the generation of the heart beat (Györke *et al.* 2007). It is established that abnormal Ca^{2+} homeostasis also plays an important role in cardiac hypertrophy and heart failure, where it has been considered either as the trigger of the hypertrophic process or as the final effector of contractile dysfunction (Frey *et al.* 2000, Carnicelli *et al.* 2008).

Usually, hypertension-induced hypertrophy and remodeling of the myocardium are mediated via angiotensin II (Ang II). Ang II plays a crucial role in modulating cardiovascular physiology and pathology by inducing signaling pathways in vascular smooth muscle cells, endothelial cells, and cardiac fibroblasts, and also by affecting their interaction with the extracellular matrix (ECM). These cascades of events, in addition to other abnormalities, ultimately lead to cardiac dysfunction in hypertension-induced hypertrophic cardiomyopathy (Mehta & Griendling 2007).

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Although some reports indicate that excess use of GC induces hypertension and cardiac abnormality, the exact mechanism of myocardial dysfunction due to this common anti-inflammatory drug has not yet been elucidated. Recently, we have demonstrated that the stimulation of Ang II signaling may be one of the mechanisms by which synthetic GC, dexamethasone (DEX), causes cardiac hypertrophy, myocardial fibrosis, hypoxia, and ventricular dysfunction (Ghose Roy *et al.* 2009). To understand further, we examined the global gene expression profile in the left ventricle of DEX-treated rat. Based on microarray results, we tested the hypothesis whether abnormal Ca^{2+} handling in cardiac myocytes is also altered due to treatment with DEX. The results suggest that altered expression of the Ca^{2+}-handling proteins and abnormal Ca^{2+} homeostasis may be responsible for myocardial dysfunction due to an excess of GC.

Materials and Methods

Materials

The synthetic GC, DEX (DEX sodium phosphate), was purchased from Naxpar Labs Ltd, India, and mifepristone (MIF; Mifegest) was purchased from Zydus Alidac, Cadila Healthcare Ltd, Ahmedabad, India. MIF and DEX of cell culture grade were purchased from Calbiochem (San Diego, CA, USA) and Sigma–Aldrich respectively. Fetal bovine serum (FBS), M199 medium, and collagenase (Type II) were purchased from Gibco BRL, New York, USA. Collagen I-coated cover glasses were obtained from BD Labware (Bedford, MA, USA). Fluo-3 acetoxymethyl ester (Fluo-3AM) was obtained from Molecular Probe (Eugene, OR, USA). Immobilon-P membranes were purchased from Millipore Corporation (Bedford, MA, USA). The Dynamo SYBR Green qPCR Kit for real-time quantitative RT-PCR was obtained from Finnzymes (Espoo, Finland). All other chemicals or reagents were obtained from Sigma–Aldrich.

Animals and administration of drugs

Male Sprague–Dawley (SD) rats (*Rattus norvegicus*) were received from the Institute's animal facility. Pups of 2 days old from the same species were taken for *in vitro* studies. The animals were handled as per the guidelines of Institutional animal ethics committee in accordance with the committee for the Purpose of Control and Supervision of Experiments on Animals, Ministry of Social Justice, Government of India. Adult male SD rats (200 g Body weight (BW)) were treated with DEX (35 μg/100 g BW, orally once daily) for 15 days. To examine the effect of excess GC on the heart, a relatively high dose of DEX was used, based on the published reports of DEX-induced cardiovascular disorders (Grunfeld & Eloy 1987, Sato *et al.* 1994, Saruta 1996). To examine the involvement of glucocorticoid receptor (GR) in mediating the cardiovascular complications, the GR antagonist, MIF (2 mg/100 g per BW, orally once daily), was co-administered (Grunfeld & Eloy 1987, Scheuer & Bechtold 2002) separately with DEX for similar periods. The rationale for terminating the studies at 15 days was that daily administration of DEX with the above for 15 days caused considerable hypertrophy, cardiac dysfunction, and other pathophysiological changes.

Assessment of cardiac hypertrophy

The rats were weighed and killed by cervical dislocation, and hearts were surgically removed and immersed in ice-cold 0·9% NaCl. The ventricles were collected, weighed, and stored at −80 °C for future use. The degree of hypertrophy was calculated in terms of heart (ventricular) weight/BW(HW/BW) ratio.

cDNA expression arrays

The analysis of global cDNA expression was carried out using the BD Atlas glass rat 3.8 I Microarray kit (Becton Dickinson, Franklin Lakes, NJ, USA). Each array consisted of long oligos for 3757 genes (organized into broad functional groups), nine housekeeping genes, and negative and positive controls, all immobilized on a glass slide. The array hybridization was performed according to the manufacturer's protocol. Briefly, 4 μg total RNA from the left ventricular tissues of control and DEX-treated rat hearts was reverse transcribed in the presence of BD powerscript reverse transcriptase and random primer mix (for synthesizing cDNA probes) at 42 °C for 60 min. The cDNA from control and DEX-treated samples was labeled using BD Atlas Powerscript Fluorescent Labeling Kit with fluorescent dye mixture of Cy3 and Cy5 (Amersham Biosciences). FluorTrap Matrix Probe Purification was performed for removal of unincorporated dye. The labeled cDNA probes were hybridized overnight at 50 °C in BD Atlas Hybridization Chamber and Glass Hybridization solution. The glass arrays were then washed four times with BD GlassHyb Wash Solution and 1X SSC. The slides were dried, and the image of the microarray was scanned with microarray laser scanner (The Packard ScanArray Express from Perkin Elmer Life Sciences, Waltham, Massachusetts, USA). The results presented are the mean ± S.E.M. of three separate microarray experiments using RNA prepared from hearts of three different batches of treatments. The list of the genes altered by DEX treatment in heart is presented as Supplementary Table 1, see section on supplementary data given at the end of this article.

Real-time quantitative RT-PCR

Levels of atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP), and a number of calcium regulatory mRNAs were measured by real-time quantitative RT-PCR using the Dynamo SYBR Green qPCR Kit and the iCycler real-time detection system and software (Bio-Rad Laboratories). For RT-PCR (20 μl), equal amounts of total RNA was used

Table 1 Oligonucleotide primer sequences used in real-time quantitative RT-PCR

Genes	NCBI Genbank accession no.	Forward primer (5′→3′)	Reverse primer (5′→3′)
ANP	NM_012612	GTGTCCAACACAGATCTGATGG	GCCAGCGAGCAGAGCCCTCA
BNP	M25297	TGGGAAGTCCTAGCCAGTCTC	GCCGATCCGGTCTATCTTCTG
GAPDH	XM_216453	GCCATCAACGACCCCTTC	AGCCCCAGCCTTCTCCA
L-type calcium channel C	NM_012517	GCTGGTGATCTTCCTGGTGT	TGAAACAGTCAAAGCGGTTG
L-type calcium channel D	NM_017298	AGGAGGAGGAAGAGGACGAG	AGACCAGGATGAGGTTGGTG
Calsequestrin	U33287	TCAAAGACCCACCCTACGTC	AGTCGTCTGGGTCAATCCAC
Triadin	NM_021666	AAGCTGAACGCCAAGAAAAA	AGCTGGTGCATCTTTGGAAT
Junctin	XM_232675	GTGGATGATGCCAAGGTTTT	TCAACACCGTCTGCAGTTTC
SERCA2a	X15635	GACGAGTTTGGGGAACAGCT	CAGGTGGTGATGACAGCAGG
Phopholamban	NM_022707	TGACGATCACAGAAGCCAAG	GTGACCCTTCACGACGATGT
Na^+/Ca^{2+} exchanger	AY033398	GAGCGCGAGGAAATGTTATC	TCCATTTTGGTTCCTCAAGC

(1 μg). Sequence-specific PCR primers were designed using the Primer 3 software (Table 1). The level of expression was calculated as fold change compared to control using the C_t value after normalizing with the housekeeping gene *GAPDH* as described earlier (Ghose Roy *et al.* 2007). The experiment was repeated with the samples from three to six different rats.

Western blot analysis

Western blot analysis was performed with left ventricular homogenates, which were prepared as described earlier (Bandyopadhyay *et al.* 2000, Ghose Roy *et al.* 2007). Briefly, the left ventricle was homogenized in a buffer containing 50 mM Tris–HCl (pH 7·4), 150 mM NaCl, 1 mM PMSF, 1 mM sodium orthovanadate, and 1 μg/ml each of pepstatin A, leupeptin, and aprotinin. Equal amount of proteins (60 μg) was resolved in 10% non-reducing SDS-PAGE for immunoblotting with anti-calsequestrin antibody (Santa Cruz Biotechnology, Inc., CA, USA). For immunodetection with antibodies for sarcoendoplasmic reticulum calcium transport ATPase 2a (SERCA2a; Santa Cruz Biotechnology, Inc.), 100 μg protein was resolved in 7·5% SDS-PAGE. The proteins were transferred to Immobilon-P membranes. The expression of actin was checked as a loading control in each experiment. For quantification, the pixel intensities of the respective bands of the immunoblots from three different individual rats were calculated and analyzed using Image J software (NIH, USA). The intensities of the bands were expressed as relative pixel density (arbitrary units).

Isolation of neonatal rat ventricular myocytes

Neonatal rat ventricular cardiomyocytes (NRVM) were prepared from 2-day-old SD rats as described earlier (Gomez *et al.* 1994, Bandyopadhyay *et al.* 2000) with some modifications. Ventricular tissue was removed and minced into small pieces in Ads buffer (116 mM NaCl, 20 mM HEPES, 0·8 mM NaH_2PO_4, 5·5 mM glucose, 5·4 mM KCl, 0·8 mM $MgSO_4$, and 100 units/ml penicillin/streptomycin, pH 7·4). Enzymatic digestion was performed in Ads buffer containing 0·05% collagenase type II and 0·25% pancreatin at 37 °C for 10 min under continuous mixing. The first tissue digest that consisted mainly of cell debris and mesenchymal cells was discarded. The 2nd–5th supernatants obtained after each 10 min digestion period were centrifuged for 5 min at 1000× ***g***. The cell pellets were re-suspended in 5 ml complete Medium 199 (M199 containing 10% FBS, 10 mM L-glutamine, and 100 U/ml penicillin–streptomycin). Following filtration through 250 μm nylon filter into complete M199, the cells were concentrated by centrifugation for 10 min at 1000× ***g***, and once more re-suspended in complete M199. For enrichment of cardiomyocytes, the cells were pre-plated on 100 mm dish for 30 min at 37 °C. The resultant suspension of cardiomyocytes was plated on collagen-coated flasks at a density of 2×10^5 cells/flask or collagen I-coated cover glasses (BIOCOAT, BD Labware, Bedford, MA, USA) and maintained at 37 °C in a humidified atmosphere containing 5% (v/v) CO_2. The majority of the cells were cardiomyocytes and beat spontaneously after 1 day of culture in complete media as reported earlier (Bandyopadhyay *et al.* 2000). Cells were stimulated with DEX (1 μM) to induce hypertrophy (Whitehurst *et al.* 1999) and MIF (5 μM) to inhibit its effect in fresh serum-free medium for 24 h. Control cells were incubated with only serum-free media under the same conditions as described above.

Measurement of Ca^{2+} transients by confocal microscopy

The free cytosolic Ca^{2+} was monitored in NRVM using the fluorescent Ca^{2+} indicator, Fluo-3AM following the protocol as described earlier (Huser *et al.* 1998, Bandyopadhyay *et al.* 2000). Briefly, cells grown on collagen I-coated cover glasses were loaded with 4 μM Fluo-3AM at 37 °C in the dark in Krebs-Ringer (KR) buffer (140 mM NaCl, 5 mM KCl, 0·5 mM $MgCl_2$, 5·5 mM HEPES, 10 mM glucose, and 1·2 mM $CaCl_2$, pH 7·4). After about 50 min, the cells were rinsed with KR buffer to remove unhydrolyzed indicator and were finally maintained for at least 15 min in an incubator to permit de-esterification of the dye. Fluo-3 fluorescence imaging was performed using Leica TCS SP2 laser-scanning

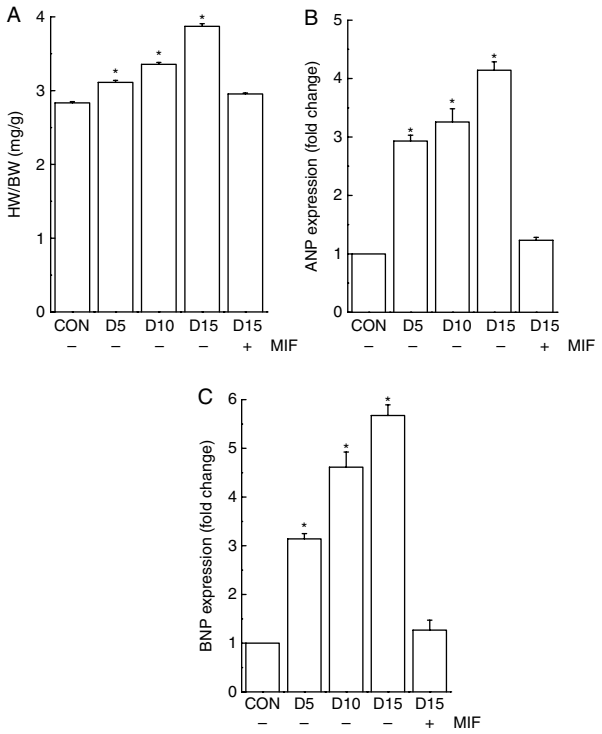

Figure 1 Induction of cardiac hypertrophy by dexamethasone. Histograms showing the degree of hypertrophy (HW/BW) of rats (A) treated with dexamethasone (DEX) for 5 days (D5), 10 days (D10), and 15 days (D15) along with regression when co-treated with mifepristone (MIF). These data represent mean ± S.E.M. for 20 rats in each group. *$P<0{\cdot}001$ versus control ($n=20$). Real-time RT-PCR showing ANP (B) and BNP (C) gene expressions as fold change (%) over control after normalizing with GAPDH mRNA expression in left ventricle of similar experimental group. Data represents mean ± S.E.M. from six animals in duplicates. *$P<0{\cdot}01$ versus control ($n=6$).

confocal microscope (Leica Microsystems, Heidelberg, GmbH, Germany). Fluo-3AM was excited with the laser at 488 nm, and fluorescence was measured at a wavelength of 515 nm as described earlier (Bandyopadhyay *et al.* 2000). The emission spectrum is expressed as normalized increase in fluorescence compared with the resting level (F/F_0). Images of contracting cells were acquired on a Leica TCS SP2 microscope, using a 63× oil immersion objective. For Ca^{2+} oscillations recording and quantitative analysis, the line-scan mode of the confocal laser-scanning microscope was used. Image acquisition (25 frames/second) and data analyses were performed using a MS Windows NT workstation with the Leica confocal software.

Statistical analysis

The data are presented as the mean ± S.E.M. Each experiment was conducted independently at least three times. For real-time PCR analysis, three to six rats were used in each group. Hypertrophy of heart was calculated based on 20 rats in each set of experiment. Data were evaluated by one-way ANOVA using the software Microcal Origin 6.0 (Microcal Software, Inc., MA, USA). A level of $P<0{\cdot}05$ was considered the threshold for statistical significance between the control and various experimental groups.

Results

Induction of cardiac hypertrophy by DEX

The treatment of rat with DEX (35 μg/100 BW) resulted in a time-dependent increase in HW/BW ratio compared to control (Fig. 1A). After 15 days of DEX treatment, about 25% increase in absolute weight of heart (combined left and right ventricle) was observed (Table 2) leading to a 36% increase in HW/BW ratio compared to control (Fig. 1A). Co-treatment of rats with the GR antagonist, MIF, along with DEX prevented hypertrophy of heart, indicating that DEX-induced cardiac growth was mediated via GR (Fig. 1A).

To confirm the induction of cardiac hypertrophy, the expression of hypertrophic markers such as ANP and BNP was also examined. The level of ANP mRNA was increased with the duration of DEX treatments attaining a fourfold induction ($4{\cdot}14 \pm 0{\cdot}14$, $P<0{\cdot}01$, $n=6$) over control after 15 days (Fig. 1B). To examine whether DEX treatment causes the development of pathological hypertrophy, the level of BNP mRNA in LV was monitored. The level of BNP mRNA was increased sixfold after 15 days of DEX treatment ($5{\cdot}67 \pm 0{\cdot}22$, $P<0{\cdot}01$, $n=6$). Co-treatment of animals with MIF prevented DEX-induced expression of BNP in LV (Fig. 1C). Taken together, the above findings demonstrated that excess of GCs resulted in the development of cardiac hypertrophy via GR.

Histological examination of hematoxylin–eosin-stained LV cross sections also showed the occurrence of myocyte hypertrophy by DEX but not in the presence of MIF (Fig. 2A and C). Sirius-stained sections through the LV showed increased deposition of collagens in the DEX-treated sample, which was mostly restored by MIF (Fig. 2B).

Altered expression of Ca^{2+} cycling/ion channel genes in DEX-induced hypertrophied heart

To examine the effect of GC treatment on cardiac gene expression, microarray analysis was performed with RNA from normal and DEX-treated rat hearts as described in

Table 2 Dexamethasone-induced cardiac hypertrophy is inhibited by mifepristone

Treatment	Control ($n=20$)	DEX ($n=20$)	DEX + MIF ($n=20$)
HW (mg)	584·2 ± 2·6	733·9 ± 7*	589 ± 2·13
BW (g)	206 ± 1	189·7 ± 1·3	199·2 ± 1
HW/BW (mg/g)	2·83 ± 0·02	3·87 ± 0·03*	2·95 ± 0·01

*$P<0{\cdot}001$ versus control, $n=20$.

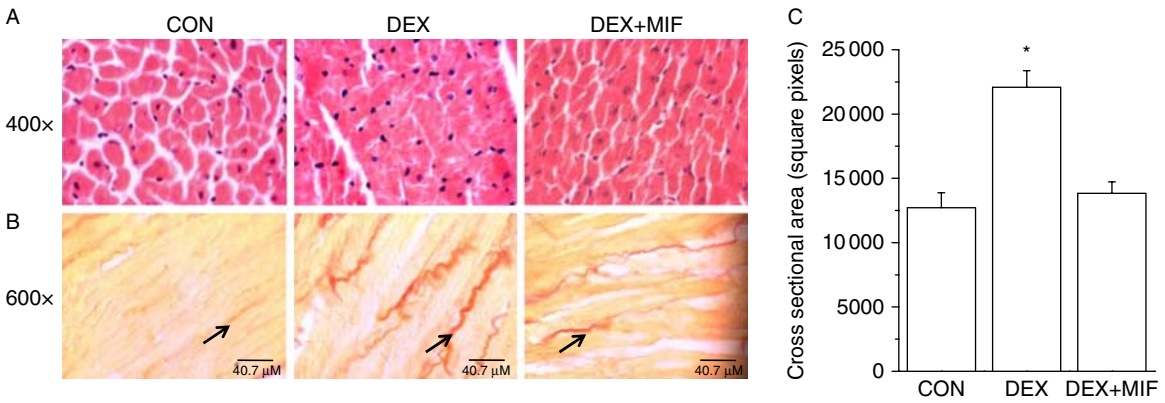

Figure 2 Development of LV fibrosis by dexamethasone. Representative images (400× magnification) showing myocyte areas in hematoxylin–eosin-stained cross sections (A) through left ventricles of rats treated with vehicle (CON) or dexamethasone (DEX) or dexamethasone with mifepristone (DEX+MIF) for 15 days. Images (600× magnification) of the similar sections (B) stained with Sirius red. Red color stretches (arrow heads) indicate collagen depositions. (C) Myocyte cross-sectional area calculated from the hematoxylin- and eosin-stained sections as shown in (A). Each bar represents myocyte cross-sectional areas in mean±S.E.M. of square pixels of three images from three individual rats (*$P<0{\cdot}01$ versus control, $n=3$). Full colour version of this figure available via http://dx.doi.org/10.1530/JOE-10-0431.

Materials and Methods. The microarray analysis of 3·4 K rat genes indicated differential expression of about 114 genes in DEX-treated heart (Supplementary Table 1). The elevated gene expression was demonstrated for about 91 genes. In contrast, only 23 genes exhibited decreased expression. The important genes of those up-regulated in DEX-treated rat heart were L-type calcium channels, calsequestrin or Ca^{2+}-sequestering ATPase, sodium–calcium exchanger, and various ion channel genes.

Effect of GC on the expression of L-type calcium channels in LV

To validate the microarray results, the levels of cardiac genes regulating Ca^{2+} homeostasis were monitored in LV homogenates. As shown in Fig. 3, DEX significantly ($P<0{\cdot}01$, $n=3$) increased mRNAs of both L-type Ca^{2+} channel C and L-type Ca^{2+} channel D with the duration (5–15 days) of treatment. There was a 3·5-fold increase in the level of L-type Ca^{2+} channel C mRNA (Fig. 3A) and a 5-fold increase in the level of L-type Ca^{2+} channel D mRNA (Fig. 3B) by DEX compared to control. Consistent with the microarray analysis, the expression of sodium–calcium exchanger was also increased by DEX treatment (Fig. 3C). The DEX-induced up-regulation of L-type Ca^{2+} channels and sodium–calcium exchanger was reversed by the GR antagonist, MIF, indicating that the GC-induced expression of these genes was mediated via GR.

Effect of GC on the expression of calsequestrin

The altered expression of calsequestrin as revealed by microarray analysis was further validated by real-time PCR as well as by western blotting analyses. As shown in Fig. 4, the level of calsequestrin mRNA was increased significantly ($P<0{\cdot}01$) with the duration of the treatment up to 15 days by DEX, which was not changed when rats were co-treated with MIF and DEX (Fig. 4A). Consistent with the mRNA level, western blot analysis showed that the protein level of calsequestrin was also increased more than twofold by DEX after 15 days compared to control which was unchanged when MIF was co-administered (Fig. 4B and C).

GC inhibits expression of SERCA2a in LV

To examine the expression of intracellular Ca^{2+}-cycling proteins further, the levels of SERCA2a and phospholamban were measured in LV homogenates. As shown in Fig. 5, the mRNA level of SERCA2a was decreased significantly ($P<0{\cdot}01$, $n=3$) in LV by DEX compared to the control. However, the level of SERCA2a mRNA was not altered by DEX when MIF was co-administered, indicating that the down-regulation of SERCA2a was not directly mediated through GR (Fig. 5A). Consistently, the protein level was also reduced by DEX (Fig. 5B), which was not restored by MIF. The level of phospholamban mRNA was marginally reduced by DEX (Fig. 5C).

DEX induces hypertrophy of cultured cardiomyocytes and alters Ca^{2+} channel genes

To examine the effect of DEX on cultured cardiomyocytes, 2-day-old NRVM were incubated in the absence or presence of DEX alone or DEX with MIF, and the expression of ANP and the genes encoding various proteins involved in Ca^{2+} homeostasis was monitored. As shown in Fig. 6A, DEX significantly ($P<0{\cdot}01$, $n=3$) increased the expression of ANP compared to control, but not in the presence of MIF. Furthermore, the mRNA level of isoforms of L-type Ca^{2+} channels ($P<0{\cdot}01$, $n=3$) was also increased by DEX (Fig. 6B and C). There was also a significant increase in calsequestrin

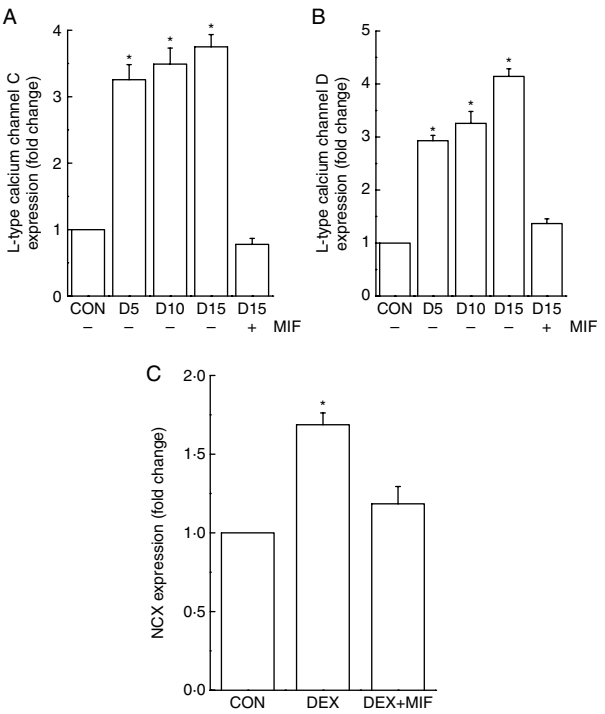

Figure 3 Expression of L-type Ca^{2+} channels and sodium–calcium exchanger on rat heart. Real-time RT-PCR analysis showing the levels of mRNA of L-type Ca^{2+} channel isoforms C (A) and D (B) in LV of rats treated with dexamethasone (DEX) for 5 days (D5), 10 days (D10), and 15 days (D15) or co-treated with DEX and mifepristone (MIF) for 15 days. The levels of mRNA of sodium–calcium exchanger in LV of rats treated with dexamethasone (DEX) or co-treated with DEX and mifepristone (MIF) for 15 days as determined by real-time RT-PCR analysis are also shown (C). Each bar indicates fold change (%) of normalized Ct values compared to control and represents mean±S.E.M. values from three animals in duplicates. *$P<0{\cdot}01$ versus control ($n=3$).

mRNA in NRVM by DEX (Fig. 6D) which was restored to the control level in the presence of MIF, indicating that the expression of calsequestrin gene was altered via GR-mediated pathway. Consistent with the results obtained with LV, the expression of SERCA2a (Fig. 6E) was significantly decreased ($P<0{\cdot}01$, $n=3$) and that of phospholamban was marginally reduced as compared to control (Fig. 6F).

Alteration of intracellular Ca^{2+} release by DEX in NRVM

Since the expression of the L-type Ca^{2+} channel proteins as well as the major regulators of the RyR/Ca^{2+} release channels was altered by DEX both *in vivo* and *in vitro*, cytosolic Ca^{2+} dynamics were monitored in NRVM cultured in the presence or absence of DEX. After 24 h of DEX treatment, the NRVM were loaded with Fluo-3AM, and both spontaneous and caffeine-induced Ca^{2+} release were monitored using laser-scanning confocal microscope. When cultured in 100% confluency, majority of the cardiomyocytes displayed spontaneous Ca^{2+} oscillations as shown in Fig. 7A. Both the frequency and the magnitude of spontaneous Ca^{2+} release were reduced substantially when NRVM were treated with DEX (Fig. 7C), but not in the presence of MIF (Fig. 7E). Application of caffeine evoked transient Ca^{2+} release in control NRVM (Fig. 7B) that was altered remarkably in DEX-treated cells (Fig. 7D). The level of cytosolic Ca^{2+} remained at the peak for a prolonged period upon stimulation with caffeine in DEX-treated NRVM (Fig. 7D). The sharp and transient Ca^{2+} release by caffeine was not fully restored when co-treated with DEX and MIF (Fig. 7F), indicating a partial restoration of the Ca^{2+}-cycling process despite the inhibition of the GR-mediated signaling by MIF.

Discussion

GCs have been widely used as an effective anti-inflammatory agent for the treatment of various diseases. However, the cardiotoxicity is one of the major side effects of GCs when used in excess. The present study demonstrates that synthetic GC, DEX, alters the expression of the ion channel genes that are important for Ca^{2+} kinetics and contractile functions of the heart. Using isolated cardiomyocytes, we further provide

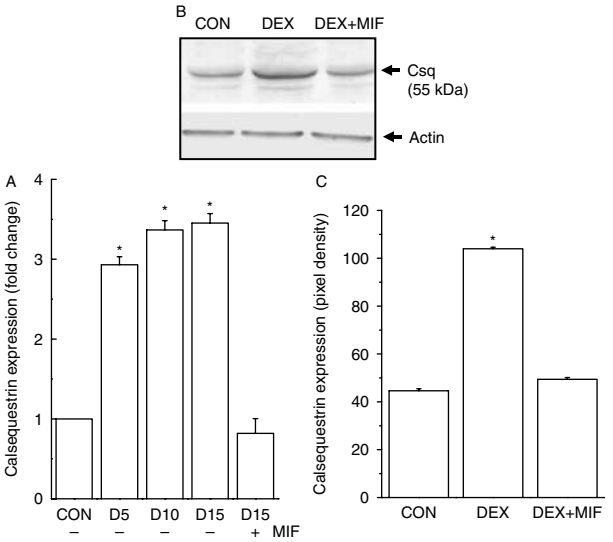

Figure 4 Dexamethasone up-regulates calsequestrin expression in heart. (A) Histograms showing the levels of calsequestrin mRNA in LV of rats treated with dexamethasone (DEX) for 5 days (D5), 10 days (D10), and 15 days (D15) or co-treated with DEX and mifepristone (MIF) for 15 days as determined by real-time RT-PCR analysis. (B) The level of calsequestrin protein in LV homogenates of rat treated in the absence or presence of DEX or co-treated with DEX and MIF for 15 days as analyzed by immunoblotting. Immunoblotting with actin antibody shows equal loading of homogenates. (C) Histogram showing mean pixel intensities (arbitrary units) of three immunoblots performed with different individual rats ($n=3$). * Significantly different compared to control at $P<0{\cdot}01$.

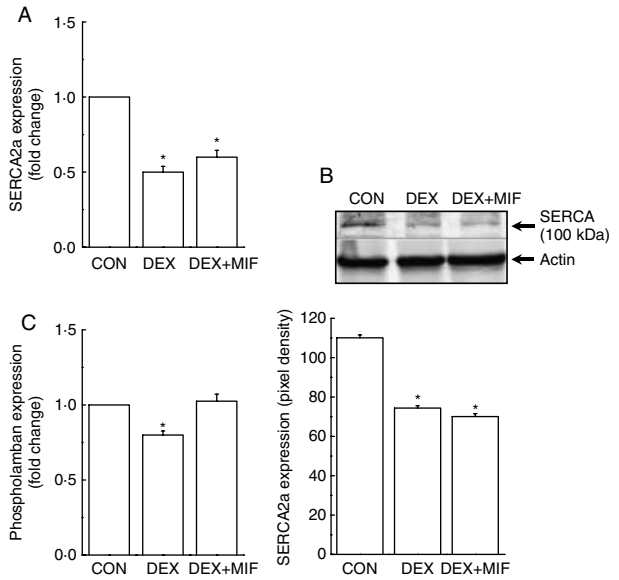

Figure 5 Effect of dexamethasone on SERCA2a and phospholamban expression. Bar graphs showing the SERCA2a mRNA (A) and protein (B) and phospholamban mRNA (C) in LV of rats treated with vehicle (CON), dexamethasone (DEX) or co-treated with DEX and mifepristone (MIF) for 15 days. Quantification of SERCA2a protein expression was performed by scanning three immunoblots from different individual rats with histograms showing as mean pixel intensities (arbitrary units). Asterisk (*) indicates significantly different compared to control at $P<0{\cdot}01$ ($n=3$). The presence of actin confirmed equal loading of the total protein.

evidence that DEX treatment may alter Ca^{2+} kinetics via altered expression of the key proteins of the ion channels, which might be responsible for cardiac malfunction resulting from treatment with excess GCs *in vivo*.

Consistent with our earlier report (Ghose Roy *et al.* 2009), we show that DEX induces cardiac hypertrophy and myocardial matrix remodeling via GR (Figs 1 and 2, Table 2). Earlier, we also reported that excess treatment of rat with DEX caused ventricular dysfunctions characterized by a significant increase in systolic pressure, slow heart beat, and decreased cardiac output (Ghose Roy *et al.* 2009). It is known that in addition to the altered ECM, abnormal Ca^{2+} handling is directly responsible for the contractile dysfunction that occurs in response to hypertension resulting into hypertrophy (Bailey & Houser 1993, McCall *et al.* 1998). Therefore, in the present study, we examined whether the alteration of Ca^{2+} signaling in cardiomyocytes was responsible for hemodynamic changes leading to cardiac dysfunction due to the treatment with DEX. The expression of a number of genes that are important for Ca^{2+} homeostasis is altered significantly in LV. The isoforms of both L-type Ca^{2+} channels and calsequestrin are increased, whereas the expression of SERCA2a is decreased considerably in DEX-treated heart. Interestingly, a direct correlation between the increase in calsequestrin and cardiac dysfunction gets supported by the previous reports, which showed that overexpression of calsequestrin in mice heart led to reduced heart function (Sato *et al.* 1998) and cardiomyopathy (Sato *et al.* 2003). Furthermore, down-regulation of SERCA2a is also reported in experimental models of cardiac hypertrophy as well as in heart failure patients (Carnicelli *et al.* 2008).

Overall, the altered expression of the key proteins involved with SR Ca^{2+} release (Figs 3–5) may lead to the dysregulation of $[Ca^{2+}]_i$ resulting in delayed relaxation and a slower heart beat in DEX-treated rat. In order to check this possibility further, we have examined the levels of those genes in NRVM (Fig. 6) and monitored the Ca^{2+} oscillations using laser-scanning confocal microscope after 24 h of DEX treatment (Fig. 7).

It is apparent that DEX alters various proteins associated with Ca^{2+} channels primarily by regulating their gene expression in LV, since the GR antagonist reversed its effects on most of the mRNAs (Figs 3–5). This is further confirmed by the results of the *in vitro* studies where it is clearly shown that DEX alters the levels of various mRNAs of the Ca^{2+} channels in NRVM also (Fig. 6). The increased expression of L-type Ca^{2+} channel isoforms by DEX is consistent with the earlier report that demonstrated that DEX triggered the

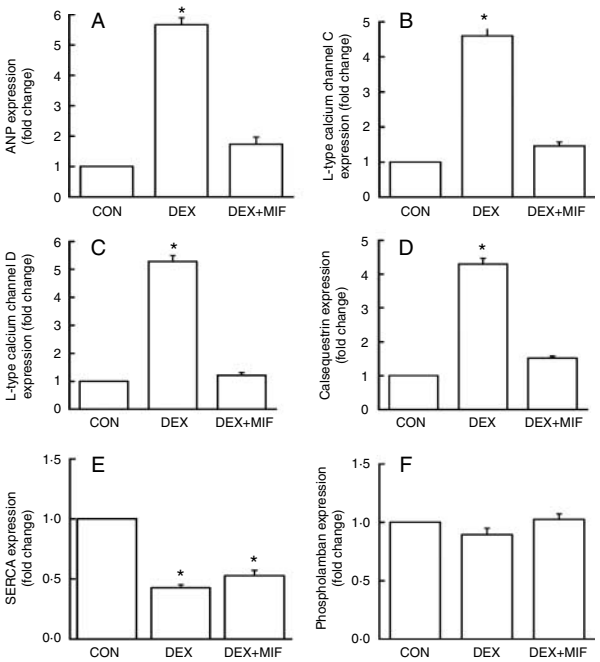

Figure 6 Levels of mRNAs for ANP and various Ca^{2+} channel genes in ventricular myocytes in response to dexamethasone. The neonatal rat ventricular myocytes were incubated in the absence (CON) or presence of 1 μM dexamethasone (DEX) or co-treated with mifepristone (DEX+MIF) for 24 h, and mRNA levels were examined by real-time PCR with gene-specific primers as described in Materials and Methods. The experiment was repeated with three different batches of cell preparations. $P<0{\cdot}01$ ($n=3$).

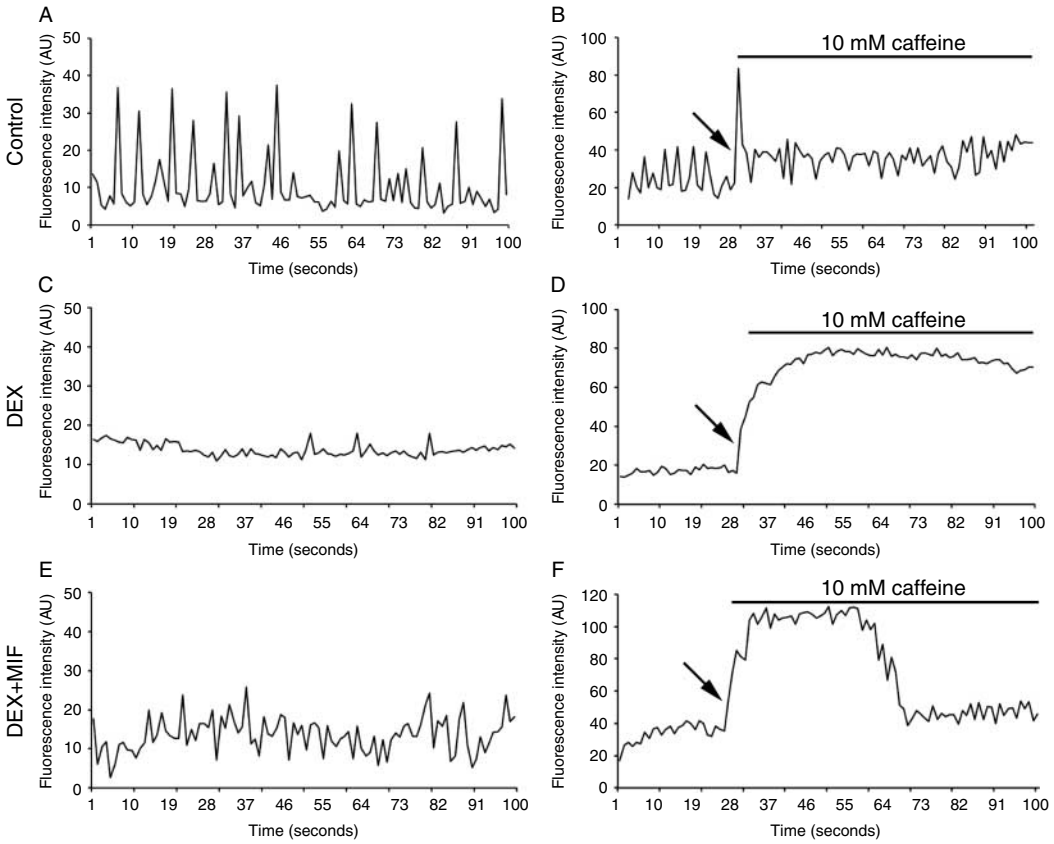

Figure 7 Effect of dexamethasone on intracellular Ca^{2+} release in NRVM. Representative traces showing Ca^{2+} release pattern in Fluo-3AM (4 μM) loaded NRVM after 24 h of incubation in the absence (A and B) or presence (C and D) of dexamethasone or dexamethasone with mifepristone (E and F). The arrow indicates the time point of caffeine addition into the chamber containing cells adhered to the coverslip. Each experiment was repeated three times, and the Ca^{2+} oscillations were recorded in ten cells each time.

expression of L-type Ca^{2+} channels due to a compensatory response to GC (Whitehurst *et al.* 1999).

In mammalian heart, Ca^{2+} release from the SR is triggered by Ca^{2+} influx via L-type Ca^{2+} channels of the sarcolemma (Wier 1990). The tendency for spontaneous activity may result from the direct influx of Ca^{2+} through L-type Ca^{2+} channels and/or occasional opening of SR Ca^{2+} release channels even under resting condition (Sitsapesan & Williams 1990, Lipp & Niggli 1994, Bandyopadhyay *et al.* 2000). In the present study, the amplitude and the frequency of the Ca^{2+} oscillations are decreased considerably in NRVM due to DEX treatment (Fig. 7). The decreased amplitude and frequency of the spontaneous Ca^{2+} oscillations may reflect the abnormal Ca^{2+} kinetics in cardiomyocytes. However, enough Ca^{2+} is stored in the SR lumen since caffeine-induced Ca^{2+} release is not affected by DEX treatment. There is a prominent difference in the pattern of transient Ca^{2+} release by caffeine in DEX-treated cardiomyocytes, which appear to be delayed and prolonged at the relaxation phase. In rat and mice ventricles, SERCA2a accounts for over 90% of the Ca^{2+} removal during cardiac relaxation (Hove-Madsen & Bers 1993, Li *et al.* 1998). Any defect in the removal of calcium from the cytosol during diastole would impair cardiac relaxation, which is critically important in that it allows the heart chambers to refill with blood in preparation for the next beat (Marks 2003).

Calsequestrin that binds Ca^{2+} with high capacity and low affinity in the SR lumen has a role as a luminal calcium sensor for the RyR. The presence of the elevated levels of calsequestrin protein slows the diffusion of Ca^{2+} in the luminal space, thereby slowing the release of Ca^{2+} through the RyR/SR channels (Terentyev *et al.* 2003, Györke *et al.* 2007). An increased Ca^{2+}-buffering capacity of the SR lumen leading to lower intraluminal free Ca^{2+} concentrations and hence lower amount of Ca^{2+} is released.

It is well known that Ca^{2+} removal from the cytosol after a caffeine response is largely dependent on the activity of Na^{+}/Ca^{2+} exchanger (Díaz *et al.* 2001). In DEX-treated heart, the expression of Na^{+}/Ca^{2+} exchanger is increased (Fig. 3C); however, cytosolic Ca^{2+} remains at the peak for a prolonged period of time upon stimulation with caffeine. It appears that the expression of Na^{+}/Ca^{2+} exchanger is not substantially

increased as compared to L-type Ca^{2+} channels and therefore fails to remove Ca^{2+} rapidly from the DEX-treated cells.

Since junctin and triadin are accessory proteins of the intracellular Ca^{2+} release channel, the expression level of these two genes was also examined. The expression of junctin mRNA in LV is marginally decreased and that of triadin remains unchanged by DEX (Supplementary Figure 1, see section on supplementary data given at the end of this article). Therefore, marginal reduction of junctin expression might also influence the cytosolic Ca^{2+} level in DEX-treated rat heart.

In contrast to reduced spontaneous Ca^{2+} oscillations in the present study, it is reported earlier that beating frequency of cardiomyocytes is increased by DEX treatment (Rossier *et al.* 2008). The exact reason for such difference is unclear, yet it is likely to be due to the difference in the method of assessment of spontaneous Ca^{2+} oscillations in two studies. The contractile activity of whole cardiomyocyte cluster as measured by Rossier *et al* (2008) may not be directly compared with the change of fluorescence intensity of Fluo-3 within the isolated cardiomyocytes in the present study.

Though the *in vivo* observations are explained on the basis of experiments conducted in the NRVM, the present study does not rule out other possibilities such as the secondary effects, if any, due to the remodeling of ECM and Ca^{2+} handling in overall reduction in heart performance.

In conclusion, it is apparent that GCs alter the expression of some of the key proteins such as L-type Ca^{2+} channels, calsequestrin, and SERCA2a, and thereby perturb the Ca^{2+} kinetics which may be implicated in cardiac abnormality. Therefore, impaired Ca^{2+} signaling may be the primary cause for cardiac dysfunction due to long-term GC therapy.

Supplementary data

This is linked to the online version of the paper at http://dx.doi.org/10.1530/JOE-10-0431.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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