Ligand-based gene expression profiling reveals novel roles of glucocorticoid receptor in cardiac metabolism

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Submitted 16 September 2008; accepted in final form 13 March 2009

Yoshikawa N, Nagasaki M, Sano M, Tokudome S, Ueno K, Shimizu N, Imoto S, Miyano S, Suematsu M, Fukuda K, Morimoto C, Tanaka H. Ligand-based gene expression profiling reveals novel roles of glucocorticoid receptor in cardiac metabolism. Am J Physiol Endocrinol Metab 296: E1363–E1373, 2009. First published March 17, 2009; doi:10.1152/ajpendo.90767.2008.—Recent studies have documented various roles of adrenal corticosteroid signaling in cardiac physiology and pathophysiology. It is known that glucocorticoids and aldosterone are able to bind glucocorticoid receptor (GR) and mineralocorticoid receptor, and these ligand-receptor interactions are redundant. It, therefore, has been impossible to delineate how these nuclear receptors couple with corticosteroid ligands and differentially regulate gene expression for operation of their distinct functions in the heart. Here, to particularly define the role of GR in cardiac muscle cells, we applied a ligand-based approach involving the GR-specific agonist cortivazol (CVZ) and the GR antagonist RU-486 and performed microarray analysis using rat neonatal cardiomyocytes. We indicated that glucocorticoids appear to be a major determinant of GR-mediated gene expression when compared with aldosterone. Moreover, expression profiles of these genes highlighted numerous roles of glucocorticoids in various aspects of cardiac physiology. At first, we identified that glucocorticoids, via GR, induce mRNA and protein expression of a transcription factor Kruppel-like factor 15 and its downstream target genes, including branched-chain aminotransferase 2, a key enzyme for amino acid catabolism in the muscle. CVZ treatment or overexpression of KLF15 decreased cellular branched-chain amino acid concentrations and introduction of small-interfering RNA against KLF15 cancelled these CVZ actions in cardiomyocytes. Second, glucocorticoid-GR signaling promoted gene expression of the enzymes involved in the prostaglandin biosynthesis, including cyclooxygenase-2 and phospholipase A2 in cardiomyocytes. Together, we may conclude that GR signaling should have distinct roles for maintenance of cardiac function, for example, in amino acid catabolism and prostaglandin biosynthesis in the heart.

endocrinology; cardiovascular system; KLF15; cylooxygenase-2; phospholipase A2

GLUCOCORTICOID HORMONES ARE ESSENTIAL FOR HOMEOSTATIC REGULATION AND PHYSIOLOGICAL MAINTENANCE OF A VARIETY OF ORGAN FUNCTIONS. CONCERNING THE HEART, NUMEROUS OBSERVATIONS HAVE SUGGESTED THAT GLUCOCORTICOIDS AS WELL AS ALDOSTERONE (ALD) HAVE BEEN SHOWN TO EXERT DIRECT EFFECTS ON CARDIOMYOCYTES AND HELP MAINTAIN VARIOUS CARDIAC FUNCTIONS. FOR EXAMPLE, IT IS SHOWN THAT A SYNTHETIC GLUCOCORTICOID, DEXAMETHASONE (DEX), SIGNIFICANTLY INCREASES THE L-TYPE Ca2+ CURRENTS (51) AND INHIBITS INDUCIBLE NITRIC OXIDE SYNTHASE ACTIVITY IN RAT CARDIOMYOCYTES (42). HOWEVER, DEX TREATMENT ENHANCES THE DEVELOPMENT OF CONTRACTILE TENSION AND INCREASES CONTRACTION AND RELAXATION VENETIONS IN CARDIAC MUSCLE (35). THE DECREASE IN CONTRACTILE FORCE OF RAT PAPILLARY MUSCLE INDUCED BY ADRENAL-CORTICOSTEROID TREATMENT IS PREVENTED BY DEX TREATMENT (27) BY MODULATING THE MEMBRANE Ca2+ AND K+ CHANNELS (33, 35, 50, 51). SHORT-TERM TREATMENT WITH DEX HAS BEEN SHOWN TO DECREASE RESTING HEART RATE IN HEALTHY HUMANS (5). IT, THEREFORE, IS APPARENT THAT GLUCOCORTICOIDS PLAY ESSENTIAL ROLES IN REGULATION OF CARDIAC ELECTRICAL AND MECHANICAL ACTIVITIES. ON THE OTHER HAND, NUMEROUS STUDIES HAVE DOCUMENTED THE PATHOLOGICAL CONSEQUENCES AND DELTERIOUS EFFECTS OF ABNORMAL OR EXCESSIVE GLUCOCORTICOID SIGNALINGS. NOT ONLY HYPERCORTISOLEMIA IN PATIENTS WITH CUSHING’S SYNDROME BUT ALSO THE CHRONIC THERAPEUTIC USE OF GLUCOCORTICOIDS IS ASSOCIATED WITH SEVERAL SIDE EFFECTS, INCLUDING ADVERSE CARDIOVASCULAR EVENTS, SUCH AS HYPERTENSION AND LEFT VENTRICULAR HYPERTROPHY (48). GLUCOCORTICOID EXCESS ALSO INDUCES METABOLIC SYNDROME WITH HYPERGLYCEMIA, DYSLIPIDEMIA, AND OBESITY, WHICH IS ASSOCIATED WITH EARLY AND PROGRESSIVE ATHEROSCLEROSIS, CONTRIBUTING TO A CLUSTER OF CARDIOVASCULAR RISK FACTORS, INCLUDING HEART FAILURE (48). MOREOVER, SHORT-TERM TREATMENT WITH DEX HAS BEEN SHOWN TO DECREASE RESTING HEART RATE IN HEALTHY HUMANS (5). IT, THEREFORE, IS APPARENT THAT GLUCOCORTICOIDS PLAY ESSENTIAL ROLES IN REGULATION OF CARDIAC ELECTRICAL AND MECHANICAL ACTIVITIES. ON THE OTHER HAND, NUMEROUS STUDIES HAVE DOCUMENTED THE DISTINCT ROLE OF GLUCOCORTICOIDS IN THE PROGNOSIS OF CARDIAC DISEASES; FOR EXAMPLE, RHEUMATOID FACTOR-POSITIVE BUT NOT NEGATIVE PATIENTS WITH RHEUMATOID ARTHRITIS WERE AT INCREASED RISK OF CARDIOVASCULAR EVENTS FOLLOWING EXPOSURE TO GLUCOCORTICOIDS (8), AND, IN PATIENTS WITH CHRONIC HEART FAILURE, HIGHER SERUM LEVELS OF CORISOL AND ALD WERE INDEPENDENT PREDICTORS OF INCREASED MORTALITY RISK (16).

Glucocorticoids and mineralocorticoids bind to the nuclear receptors glucocorticoid receptor (GR) and mineralocorticoid receptor (MR), both of which are transcription factors and expressed in cardiomyocytes. However, the role of these receptors in cardiac physiology remains elusive. Indeed, the ligand-receptor interactions are complicated, as both ALD and glucocorticoids can activate cardiac MR, thereby directly affecting heart function (48). Some of the cardiac or peripheral effects of glucocorticoids may be mediated at least in part by MR activation. In “classical” ALD target cells (i.e., kidney and colon), MR is protected from illicit occupation by glucocorti-
coids because of the presence of 11β-hydroxysteroid dehydroge- 

nase type II (11βHSD2), an enzyme that converts cortisol 

(human)/corticosterone (COR, rodents) into inactive metabo- 
lites. Cardiomyocytes belong to the so-called “nonclassical” 

ALD target tissues that express both GR and MR, but not 

11βHSD2. In cardiomyocytes, thus, MR is not protected from 
occupancy by glucocorticoids and is not ALD selective. Tak- 
ing into account that circulating cortisol/COR levels are at least 

100-fold higher than those of ALD, and that MR has the same 

affinity for ALD and glucocorticoids, MR, as well as GR, may 

be permanently occupied by glucocorticoids, and glucocorti- 
coid effects could be mediated by both GR and MR (48). The 

recent advent of microarray and other technologies has facil- 
titated the identification of a number of glucocorticoid-regulated 
genes (1, 20, 34, 36, 45), and it becomes apparent that the 
profile of those glucocorticoid-target genes differs according to 
the cell types and the mode of interaction with ligands (49). 
However, because of the redundancy of the ligand-receptor 
interaction, not a single study could clearly differentiate target 
genes for cardiac GR and MR. Recently, a transgenic mouse 
model with conditionally inducible cardiac-specific expression 
of human GR was generated to preclude secondary effects due 
to general glucocorticoid-induced alterations and to investigate 
the specific role of GR in cardiomyocytes, and electrophysio- 
logical phenotyping indicated that cardiac GR overexpression 
resulted in conduction defects, with high-degree atrio-ventric- 
ular block (39). These results strongly support such an idea that 
GR has as yet unknown but essential roles in the heart. It, 
therefore, is important to delineate how these nuclear receptors, 
especially GR, differentially couple with ligands and regulate 
gene expression for operation of their distinct functions in 
cardiomyocytes.

We previously reported that a synthetic glucocorticoid, cor- 
tivazol (CVZ), could be extremely specific for GR and does not 
crossreact with MR (52, 53). Given this, we indicated that a 
ligand-based approach involving CVZ and GR antagonist RU-

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crossreact with MR (52, 53). Given this, we indicated that a 
ligand-based approach involving CVZ and GR antagonist RU-

486 might be applied to define the role of GR in nonclassical 
ALD target tissues. In the present study, we performed mi-
croarray analysis based on this ligand-based approach and 
differentially characterized corticosteroid target genes, and 
the distinct role of GR in cardiomyocytes was discussed.

MATERIALS AND METHODS

Reagents and antibodies. CVZ was kindly gifted from Sanofi-

Aventis (Paris, France). COR, ALD, interleukin (IL)-1β, lipopolysac-
charide, estradiol, progesterone, and RU-486 were purchased from 
Sigma (St. Louis, MO). MG-132 was purchased from Calbiochem 
(San Diego, CA). Other reagents were from Nacalai Tesque (Kyoto, 
Japan) unless otherwise specified. Anti-GR (sc-1004), anti-cyclooxy-
genase-2 (COX-2, sc-1747), and anti-KLF15 (sc-34827) antibodies 
were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). 
Anti-α-actin (A7811) and anti-FLAG (F1804) antibodies were ob-
tained from Sigma.

Plasmids, small-interfering RNA oligonucleotides, and recombi-
nant adenoviruses. To construct the expression plasmids for FLAG-
tagged rat GR and MR, either full-length cDNAs for rat GR or MR 
were inserted in p3xFLAG-CMV10 vector (Sigma). The glucocorti-
coid response element (GRE)-driven reporter plasmid p2xGRE-LUC 
was described previously (52). Small-interfering RNA (siRNA) oli-

gonucleotides against rat GR (Silencer Predesigned siRNA ID: 
199951) and control siRNA (Silencer Negative control siRNA no. 1: 
07606954A) were purchased from Ambion (Austin, TX). siRNA 
oligonucleotides against rat KLF15 (Stealth Select RNAi RS340443) 
were purchased from Invitrogen (Carlsbad, CA). Recombinant aden-

oviruses encoding FLAG-tagged rat KLF15 and Cre-recombina 

were generated by using the Adenovirus Cre/IoxP-regulated Expression 
Vector Set (TaKaRa, Otsu, Japan) as per the manufacturer’s instruc-
tions and as previously described (44). Recombinant adenoviruses 
prepared from 293 cells were purified with Viratik AdenoMini-24 
(Virapur, San Diego, CA) and titrated using an Adeno-X Rapid Titer 
Kit (TaKaRa).

Cell culture. COS-7 cells were obtained from RIKEN Cell Bank 
(Tsukuba, Japan) and cultured in DMEM (Invitrogen) supplemented 
with 10% FCS (Sigma-Aldrich, St. Louis, MO) and antibiotics in a 
humidified atmosphere at 37°C with 5% CO2. Primary cultures of 
cardiomyocytes were prepared as described previously (40). In brief, 
the ventricles of 1-day-old neonatal Wistar rats (CLEA Japan, Tokyo, 
Japan) were dissociated in 0.03% trypsin, 0.03% collagenase, and 20 
µg/ml of DNase I. The cardiomyocytes and cardiac fibroblasts were 
separately prepared on the basis of their differential adhesiveness. 
Attached cells (mostly cardiac fibroblasts) were subcultured two times 
to deplete cardiomyocytes, and the third passage cells were used. 
CARDIOMYOCYTES were seeded at a density of 1 × 10⁵ cells/cm² on 
gelatin-coated dishes and grown in medium 199/DMEM (Invitrogen) 
supplemented with 10% FCS and antibiotics in a humidified atmo-
sphere at 37°C with 5% CO2. Concerning animal experiments, all 
procedures and protocols were approved by the Animal Care and Use 
Committee of Keio University.

Immunofluorescence. FLAG-tagged rat GR- or MR-expressing 
COS-7 cells were plated on glass coverslips in a six-well plate. Fixed 
and permeabilized cells were blocked with blocking buffer (3% BSA 
and 0.1% Triton X in Tris-buffered saline). The cells were then 
stained with primary antibodies against FLAG (1:500) for 1 h at room 
temperature, and then, secondary antibodies conjugated with Alexa 
Fluor 488 (1:500, Invitrogen) were applied for 1 h at room tempera-
ture. The stained cells were observed by confocal laser scanning 
microscopy (LSM510; Carl Zeiss, Jena, Germany) with appropriate 
emission filters.

Western blotting. Whole cell extracts were prepared in Nonidet 
P-40 (NP-40) lysis buffer (150 mM NaCl, 1.0% NP-40, 50 mM Tris, 
PH 8.0, and protease inhibitor cocktail) and boiled in SDS sample 
buffer, analyzed by SDS-PAGE, and electrophoretically transferred to 
a polyvinyl difluoride membrane (Millipore, Bedford, MA). Subse-
quently, immunoblotting was performed with anti-GR, anti-α-actin, 
anti-KLF15, anti-FLAG, or anti-COX-2 antibodies diluted at 1:1,000, 
followed by horseradish peroxidase-conjugated secondary antibodies 
(Amersham Biosciences, Buckinghamshire, UK) diluted at 1:2,000. 
Antibody-protein complexes were visualized using the enhanced 
chemiluminescence method according to the manufacturer’s protocol 
(Amersham Biosciences). Signal intensity of the band for GR relative 
to that for α-actin was quantified using the analysis software from 
the National Institutes of Health (NIH image 1.62).

Transfection and reporter gene assay. Transient transfection 
and reporter gene assay were performed as described previously (53). In 
brief, cells were plated on 6-cm-diameter culture dishes, and cell 
culture medium was replaced with serum-free medium OPTI-MEM 
lacking phenol red (Invitrogen) before transfection. Plasmids or 
siRNA oligonucleotides were mixed with Lipofectamine 2000 trans-
fection reagent (Invitrogen) and added to the culture according to the 
manufacturer’s protocol. The total amount of the plasmids was kept 
constant by adding an irrelevant plasmid (pGEM3Z was used unless 
otherwise specified). After 6 h of incubation, the medium was re-
placed with fresh OPTI-MEM, and the cells were further cultured in 
the presence or absence of various reagents for 24 h at 37°C. In 
reporter gene assay, whole cell extracts were prepared in Cell Culture 
Lysis Reagent (Promega, Madison, WI) on ice for 15 min followed by 
centrifugation for 20 min at 20,000 g. Luciferase enzyme activity was 
determined using the Luciferase Assay System (Promega) and a 
luminometer (Promega) according to the manufacturer’s protocol.
Relative light units were normalized to the protein amounts determined with BCA Protein Assay Reagent (Pierce, Rockford, IL).

**Microarray analysis.** Primary cultures of cardiomyocytes, grown in serum-free medium OPTI-MEM for 24 h, were treated with vehicle (ethanol) or various ligands for 3 h with or without pretreatment of 10 μM RU-486. Total RNA was isolated using TRIZOL-Reagent (Invitrogen) according to the manufacturer’s protocol and further purified using the RNeasy Mini Kit (Qiagen, Valencia, CA). We used pooled RNA samples from three independent experiments, and DNA microarray analysis were performed two times as follows: first experiment using the RNeasy Mini Kit (Qiagen, Valencia, CA). We used vitrogen (according to the manufacturer’s protocol and further purified by using a GeneChip Sample Cleanup Module (Affymetrix). In vitro transcription reactions were performed using a GeneChip IVT Labeling Kit, which includes T7 RNA polymerase and Biotin-labeled ribonucleotides. Biotin-labeled cRNA was purified using a GeneChip Sample Cleanup Module. The concentration of cRNA was calculated from light absorbance at 260 nm using an ultraviolet spectrophotometer. Next, cRNA (15 μg) was hybridized to the Affymetrix GeneChip Rat Genome 230 2.0 Array (Affymetrix), on which 31,099 probe sets and 12,379 gene sets are represented. The array was incubated for 16 h at 45°C and then automatically washed and stained with the GeneChip Hybridization, Wash and Stain Kit (Affymetrix). The Probe Array was scanned using a GeneChip Scanner 3000 7G. The raw data were normalized and analyzed using GeneChip Affymetrix GCOS 1.2 software and GeneSpring 7.3.1 (Agilent Technologies, Palo Alto, CA). In per-chip normalization, a raw intensity value was divided by the median value of the chip measurements, and then, each gene was normalized to the respective control to enable relative changes in gene expression levels between samples. The signal values and the present (P flag), absent (A flag), or marginal (M flag) calls were computed for all probe sets, and only probe sets with the present calls were used in the further analysis. Only the significantly expressed genes in both experiments were considered to be valid, and Ingenuity Pathway Analysis (http://www.ingenuity.com; Ingenuity Systems, Redwood City, CA) was used to map those probes to genes with annotation, to perform pathway analysis, and to create gene networks. Functional classifications according to Gene Ontology (GO) terms were performed by using ExPlain (BIOSABE, Wolfenbüttel, Germany, www.biobase.de). The data discussed in this publication have been deposited in the National Center for Biotechnology Information’s Gene Expression Omnibus [GEO (11)] and are accessible through GEO Series accession no. GSE12752 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE12752).

**Real-time quantitative RT-PCR.** Total RNA from primary cultures of cardiomyocytes was reverse-transcribed with oligo(dT) primers using the SuperScript III First-Strand Synthesis Kit for RT-PCR (Invitrogen). Real-time quantitative RT-PCR (qRT-PCR) was performed with the LightCycler TaqMan Master, Universal ProbeLibrary Set, Rat, and LightCycler ST300 systems (Roche, Basel, Switzerland) according to the manufacturer’s instructions. Relative expression levels were calculated on the basis of an internal standard curve generated for each gene, and mRNA for glyceraldehyde-3-phosphate dehydrogenase (Gapdh) was used as an internal control. The primer sequences used in this study are as follows: *Gapdh*: 5'-agccacatgtgtctcag-3' and 5'-gctggattcataactcctct-3'; *Klf15*: 5'-ctgctgagaggtctcagc-3' and 5'-gctggattcataactcctct-3'; *Bcat2*: 5'-agtggctgtcctcag-3' and 5'-ccatgctcatcagctg-3'; *Slc2a4* (glucose transporter 4, GLUT4): 5'-tgatggctcagctgtctcct-3' and 5'-acagctgctgtctcctcct-3'; *Foxol*: 5'-tcagctggtgctgtctcct-3' and 5'-gctgtgctgtctcctc-3'; *Ptgs2* (myostatin): 5'-gctggattcctggtctcctc-3' and 5'-gtgtgctgtctcctc-3'; *Gdf8* (myostatin); 5'-tgcctgcattgttcagc-3' and 5'-tgtaattctcctcctc-3'; *Sgk1*: 5'-tccccctctggctggaagc-3' and 5'-ttttgctgtctcctcctc-3'; *Npph* (brain natriuretic peptide, BNP): 5'-gctggattcctcctcctcctc-3' and 5'-gctggattcctcctcctcctc-3'; *Pla2g4a* (cytoplasmic phospholipase A2, PL2A): 5'-tcctgctgtcctcctcctc-3' and 5'-gagctgctgtcctcctcctc-3'.

**Measurement of amino acid concentration.** Measurement of amino acid concentration of cultured neonatal rat cardiomyocytes was performed as described previously (19) with minor modification. In brief, after medium replacement to the serum-free medium OPTI-MEM, cultured neonatal rat cardiomyocytes were infected or transfected with KLF15-expressing adenoviruses or siRNA oligonucleotides, respectively, and cultured for 24 h. The medium was replaced to fresh OPTI-MEM, and the cells were further cultured with or without CVZ, ALD, or COR for 24 h. The cells were washed three times with PBS and lysed in 1 ml of ice-cold methanol for 5 min, except for the dish with the same protocols for counting the number of cells. Cellular lysates and recovery efficiency control Phe-d5 were collected in 15-ml tubes, 1 ml of chloroform was added to the lysates, and the mixtures were briefly vortexed. The mixtures were centrifuged at 1,000 g, 4°C for 5 min, and the supernatants were transferred to new 15-ml tubes. This chloroform precipitation method was again repeated, and the supernatants were concentrated and dried with a AES2010 SpeedVac system (Savant Instruments, Holbrook, NY) and resuspended in 200 μl of MilliQ ultra pure water (Millipore). Quantification of collected amino acid was performed with high-performance liquid chromatography-tandem mass spectrometry assay using Agilent 1100 HPLC (Agilent) interfaced to an Applied Biosystems/Sciex API 4000 triple quadrupole mass spectrometer (Applied Biosystems, Foster City, CA). Data collection and processing were performed with Sciex Analyst version 1.4.2 software (Applied Biosystems).

**Statistical analysis.** Except for DNA microarray analysis, we performed all experiments in triplicate, and the results are expressed as means ± SE of three independent experiments as indicated. The statistical significance of differences between groups was calculated either by one- or two-way ANOVA, and the difference was considered significant at P < 0.05.

**RESULTS AND DISCUSSION**

**GR in rat cardiomyocytes and its ligand specificity.** At first, to verify the feasibility to use isolated rat cardiomyocytes for identification of GR target genes, the presence of GR was confirmed in Western blot analyses. As shown in Fig. 1A, ligand-dependent nuclear localization of endogenous GR was clearly demonstrated in the presence of either endogenous or synthetic corticosteroids, COR and ALD, or CVZ, respectively, at the concentration of 100 nM for 1 h. Moreover, ligand-activated GR was shown to be able to induce expression of GRE-driven luciferase reporter gene (Fig. 1B). ALD, as previously reported (2, 38), appeared to be a weaker agonist compared with the other two glucocorticoids, since proportions of nuclear-translocated GR (Fig. 1A) and transactivation potential (Fig. 1B) were relatively smaller. We previously characterized CVZ as a GR-specific ligand without MR activation capacity (see introduction). To test whether this is also the case in rat GR and MR, we transfected the expression plasmids for rat GR or MR together with GRE-luciferase reporter gene in COS-7 cells. After treatment with 100 nM of COR or ALD, both rat GR and MR translocated in the nucleus. However,
CVZ failed to promote nuclear translocation of not GR but MR (Fig. 1C). This issue is further supported by the luciferase assay in which CVZ again failed to induce MR-dependent reporter gene activation (Fig. 1D). It was also shown that the GR antagonist RU-486 shut down GR-dependent GRE-lucerase reporter gene activation by either CVZ, COR, or ALD; however, RU-486 did not repress ALD or COR-inducible MR-dependent reporter gene activation (Fig. 1, B and D). We, therefore, concluded that CVZ and RU-486 are useful to differentiate GR-dependent gene expression profile from that of MR as GR-specific agonist and antagonist, respectively.

Global analysis of gene expression after treatment with corticosteroids in rat cardiomyocytes. To identify which set of gene expression is influenced by GR, we analyzed gene expression changes after exposure of cells to COR, ALD, and CVZ in the absence or presence of RU-486. Because our preliminary experiments using several cell lines showed that expression of many GR target genes was induced by COR at the concentration of 100 nM in 3 h and previous reports indicated that a concentration of 100 nM of COR was considered to be equivalent to maximal and supraphysiological level in cultured cells (13, 22), we in the present study set the concentration of these ligands and the time periods of exposure as 100 nM and 3 h, respectively. We also expected that this relatively short exposure would avoid secondary effects of the products of GR-regulated genes. The results of our microarray analyses were summarized in Fig. 2 and Table 1 [the detailed results were uploaded in Supplemental Table 1 (Supplemental data for this article can be found on the American Journal of Physiology-Endocrinology and Metabolism website)]. Among 12,379 genes, 7,351, 7,478, 7,507, 7,803, 7,863, and 7,845 genes were considered to be relevant for further analysis for CVZ-, COR-, ALD-, CVZ + RU-486-, COR + RU-486-, and ALD + RU-486-treated cells, respectively (see MATERIALS AND METHODS for details). Four hundred genes were significantly induced, and 57 genes were repressed after treatment with either CVZ, COR, or ALD (Table 1). For classification, a Venn diagram was applied, and it was revealed that treatment with CVZ, COR, and ALD induced 351 (categories 1, 4, 6, and 7), 192 (categories 2, 4, 5 and 7), and 87 (categories 3, 5, 6, and 7) genes, respectively, with significant overlap between each (Fig. 2A). RU-486 sensitivity of the genes in categories 1, 4, and 7 was 91.1% (for CVZ), 95.1% (for COR), and 90.5% (for ALD) and 91.1% (for CVZ), 94.6% (for CVZ), and 75% (for COR), respectively (Table 1). We, therefore, may indicate that expression of the majority of those genes induced by CVZ or COR in categories 1, 4, and 7 was considered to be mediated through GR. Indeed, the gene set that was induced by CVZ and COR (categories 4 and 7) contained many classical glucocorticoid-regulated genes, e.g., PDK4, SGK, and FKBPs, and the fold inducibility appeared to be greater in CVZ than in COR or ALD (Supplemental Table 1). When CVZ and COR were compared, 159 genes were induced by both CVZ and COR, corresponding to categories 4 and 7, but 192 genes (54.7% of CVZ-induced genes, corresponding to categories 1 and 6) were induced not COR but by CVZ, and 33 (17.2% of COR-induced genes, corresponding to categories 2 and 5) were induced by COR but not CVZ. Considering that CVZ has stronger agonistic activity compared with COR or ALD, it was unexpected that these 33 genes (category 2 and 5) were not induced by CVZ. RU-486 sensitivity of those 33 genes belonging to categories 2 and 5 was slightly lower (60.6% for COR) than that of 192 genes of categories 1 and 6 (89.5% for CVZ). Concerning the genes in categories 2 and 5, fold inducibility by COR was marginal, and RU-486 sensitivity was equivocal (Supplemental
Concerning ALD action, mRNA expression of 87 genes was induced by ALD (categories 3, 5, 6, and 7). Among 384 genes that were induced by either CVZ or COR (categories 1, 2, 4, 5, 6, and 7), only 71 genes (18.5% of 384 genes) were induced by ALD (81.6% of 87 ALD-induced genes). When the genes belonging to category 7 were excluded, we could not find known glucocorticoid-regulated genes in the ALD-induced gene set. Moreover, fold inducibility of the majority of ALD-induced genes appeared to be marginal (Supplemental Table 1), and RU-486 sensitivity was relatively low (50, 66.7, 50, 41.1% for ALD, in categories 3, 5, 6, and 7, respectively) (Table 1). We, thus, may consider that, at least as far as a number of induced genes and their fold inducibility, glucocorticoids appear to be a major determinant of GR-mediated gene expression in cardiomyocytes.

The total number of downregulated genes ($n = 57$) was smaller than that of upregulated genes ($n = 400$) in rat cardiomyocytes, and again CVZ appeared to be stronger than COR or ALD (Supplemental Table 1 and Fig. 2B). In clear contrast to transcriptional induction, RU-486 is known to have a similar transrepressive effect when compared with agonistic glucocorticoids, including CVZ and COR (17). The ligand-based approach, therefore, did not appear to be merited in further analysis of those repressed genes, and we focused on the induced genes in the following sections.

**GO analysis of corticosteroid target genes.** Results of GO analysis were represented as boxed charts in Fig. 2C. The pattern of the charts was similar between CVZ-induced genes and COR-induced ones; these ligands influenced such genes belonging to, for example, protein modification/metabolism, cell differentiation, nucleic acid metabolism, transcription, apoptosis, and lipid metabolism. However, the number of genes in each category was drastically different between CVZ-induced genes and COR-induced ones (Fig. 2C). We (52, 53) and others (49) previously indicated that CVZ may have distinct target gene sets when compared with natural glucocorticoids, since CVZ has a phenylpyrazol moiety at the A ring of steroid structure. Indeed, Miller et al. (32) also revealed that, while CVZ and DEX overlap in regulation of most genes, each steroid regulates expression of an exclusive set of transcripts in CEM-C7-14 cells (sensitive to apoptosis by both DEX and CVZ) and CEM-C1-15 cells (DEX-resistant but CVZ-sensitive). Moreover, they showed that 57 genes were regulated uniquely to a statistically significant extent by CVZ in both clones and many of the CVZ specific genes are key components of various signal transduction pathways and not all but some are related to apoptosis. The fact that the order of the number of induced genes was CVZ $>$ COR $>$ ALD in our study may support such an idea that CVZ may have a distinct target gene set.

These gene expression profiles suggested numerous roles of corticosteroids in various aspects of cardiac physiology and that glucocorticoids and mineralocorticoid, and GR and MR as well, appeared to have distinct sets of target genes in cardiomyocytes. For example, among others, corticosteroids induced mRNA expression of FKBP5 via GR, in the descending rank order of CVZ, COR, and ALD, with efficient suppression by RU-486 (Supplemental Table 1). It, therefore, may be concluded that FKBP5 gene expression is driven by the glucocorticoid-GR axis. Because FKBP5 is shown to be contained in the GR chaperon complex with heat shok protein-90, this result may indicate that the ultrashort feedback loop of GR operates to induce myocardial hypertrophy in vivo, however, and the effects of glucocorticoids on the cell size of cardiomyocytes are still controversial in vitro (10, 14, 26, 51). Indeed, several reports have suggested that treatment of cardiomyocytes with COR alone has had a little effect for the cell growth and enlargement (24, 28). In our experimental settings, DNA microarray and qRT-PCR analysis revealed that, in cultured cardiomyocytes, CVZ and COR induced mRNA expression of several prohypertrophic genes such as SGK and BNP (Supple-
Table 1. Classification of corticosteroid-induced and -repressed genes in DNA microarray analysis

<table>
<thead>
<tr>
<th>Category</th>
<th>Total</th>
<th>CVZ RU sensitive</th>
<th>CVZ RU insensitive</th>
<th>COR RU sensitive</th>
<th>COR RU insensitive</th>
<th>ALD RU sensitive</th>
<th>ALD RU insensitive</th>
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No. of genes grouped by the category classified in Venn diagrams as shown in Fig. 2 together with the presence or absence of antagonism by RU-486 (RU-sensitive or -insensitive, respectively) for upregulated (>1.5-fold) or downregulated (<0.5-fold) genes in DNA microarray analysis as described in MATERIALS AND METHODS. RU-486-sensitive, ([X + RU-486] − 1)/([X] − 1) < 0.5; RU-insensitive, ([X + RU-486] − 1)/([X] − 1) > 0.5 [X is either cortivazol (CVZ), corticosterone (COR), or aldosterone (ALD), and square brackets depict fold induction in Supplemental Table 1].

mental Table 1 and Fig. 3). In contrast, CVZ and COR also induced mRNA expression of atrophy-related genes, i.e., FOXO1a, atrogin-1, and myostatin (Supplemental Table 1 and Fig. 3), which are known as the regulators of muscle mass via the ubiquitin-proteasome pathway (30). CVZ or COR treatment of cultured cardiomyocytes for 72 h did not significantly affect their cell size (data not shown). Together, it is indicated that glucocorticoids have distinct sets of target genes in cardiomyocytes, and, among them, balance between prohypertrophic genes and proapoptotic genes might, at least in part, determine cell size. Such balance might be regulated not only by glucocorticoids but also by various extra- and/or intracellular factors, e.g., hypertension and metabolic status. Indeed, it has been reported that glucocorticoid-induced cardiac enlargement of the rat heart was transient, and extension of treatment duration with a high level of glucocorticoid brought about anabolic to catabolic state transformation with the loss of the cardiac growth (6, 25).

Of note, it was revealed that glucocorticoids induce mRNA expression of numerous transcription factors, including FOXO1a, C/EBPβ, PGC-1α, and a member of Kruppel-like transcription factors KLF9 and KLF15 (Supplemental Table 2). Their induction response was greater in CVZ and COR than in ALD and significantly repressed by RU-486, and their mRNA expression is also considered to be transcriptionally regulated by GR (Supplemental Table 2). Because not all but many of them are known to be involved in various metabolic processes (9), our results may indicate that glucocorticoid-GR modulates complex metabolic milieu via cascade of regulation of gene expression in the heart.

Fig. 3. Time course of mRNA expression of glucocorticoid-regulated genes in rat cardiomyocytes. Total RNA was isolated from neonatal rat cardiomyocytes after treatment with 100 nM CVZ (circles), COR (squares), or ALD (triangles) for the indicated time periods, and was analyzed in qRT-PCR as described in MATERIALS AND METHODS. mRNA expression levels were normalized to glyceraldehyde-3-phosphate dehydrogenase (Gapdh) and relative expression levels to the 0-h samples are presented. Means ± SE of 3 independent experiments are shown. BNP, brain natriuretic peptide. *P < 0.05 vs. cells treated with each ligand at 0 h.
considered to be involved in amino acid catabolism to induce branched-chain aminotransferase 2 (BCAT2) gene expression, which is rate-limiting for amino acid breakdown in skeletal muscle and increases alanine production for liver gluconeogenesis (12).

We showed that glucocorticoids induce mRNA expression of KLF15 in cardiomyocytes. This issue was further supported by qRT-PCR analysis and siRNA experiments; after treatment with not ALD but CVZ or COR in cardiomyocytes, mRNA expression of KLF15 was rapidly increased (from 3 h after treatment with corticosteroids) in a time- and concentration-dependent manner (Fig. 4, A and B). Moreover, such induction response was cancelled by introduction of siRNA against GR (Fig. 4C), indicating that mRNA induction of KLF15 is mediated through GR. It is known that gene expression of BCAT2 and GLUT4 is transcriptionally controlled by KLF15 (12, 15). We showed that mRNA expression of BCAT2 and GLUT4 (Slc2a4) genes was increased after treatment with CVZ and COR with a lag time of ~3–6 h after apparent induction of KLF15 mRNA in cardiomyo-

Fig. 4. KLF15 is a GR target gene involved in the amino acid catabolic pathway in rat cardiomyocytes. A: time course of mRNA expression of KLF15 and its target genes. Total RNA was isolated from neonatal rat cardiomyocytes after treatment with 100 nM of CVZ (circles), COR (squares), or ALD (triangles) for the indicated time periods and was analyzed in qRT-PCR as described in MATERIALS AND METHODS. mRNA expression levels were normalized to Gapdh, and relative expression levels to the 0-h samples are presented. Means ± SE of 3 independent experiments are shown. *P < 0.05 vs. cells treated with each ligand at 0 h. B: concentration-dependent regulation of KLF15 gene expression by glucocorticoids. Total RNA was isolated from neonatal rat cardiomyocytes after treatment with the indicated concentrations of CVZ (circles) or COR (squares) for 3 h. mRNA expression levels were normalized to Gapdh, and relative expression levels to the vehicle-treated samples are presented. Means ± SE of 3 independent experiments are shown. *P < 0.05 vs. cells treated with vehicle. C: effect of GR knockdown on glucocorticoid-dependent induction of mRNA expression of KLF15 and BCAT2. The cardiomyocytes were transfected with control small-interfering RNA (siRNA) or siRNA oligonucleotides for GR, KLF15 as indicated, and cultured for 24 h. Next, the cells were treated with vehicle or 100 nM CVZ for 12 h, and total RNA was analyzed with qRT-PCR. Results were normalized to Gapdh, and relative expression levels to vehicle-treated samples are presented. Means ± SE of 3 independent experiments are shown. *P < 0.05 vs. vehicle-treated cells. D: Western blot analysis for KLF15 protein. Control siRNA, siRNA against GR (si-GR), siRNA against KLF15 (si-KLF15), Cre-expressing adenoviruses [Ad-Cre, multiplicity of infection (MOI) = 5], and floxed FLAG-tagged KLF15-expressing adenoviruses (Ad-KLF15, MOI = 10) were introduced in cardiomyocytes and were cultured in the presence or absence of 5 μM MG-132 for 12 h as indicated. Next, whole cell extracts were prepared, and Western immunoblot was performed with anti-KLF15 antibodies (left) and anti-FLAG antibodies (middle). Experiments were repeated 3 times with almost identical results, and representative photographs are shown. E: induction of mRNA expression of BCAT2 and GLUT4 by KLF15. Ad-Cre and Ad-KLF15 were infected in rat cardiomyocytes as indicated, and the cells were cultured for 24 h. Total RNA was prepared and analyzed with qRT-PCR. Results were normalized to Gapdh, and results are expressed as relative expression levels to Ad-KLF15(−) cells. Means ± SE of 3 independent experiments are shown. *P < 0.05 vs. Ad-KLF15(−) cells.
cytes (Fig. 4A). In addition, the fact that siRNA for either GR or KLF15 shut down hormone-dependent induction of BCAT2 mRNA expression (Fig. 4C) strongly argues the critical importance of the glucocorticoid-GR-KLF15 pathway for BCAT2 gene expression.

Next, we further addressed the role of GR-dependent KLF15 induction in cardiomyocytes. In Western blot analysis, the KLF15 protein band was not detected after treatment of cardiomyocytes with CVZ alone. However, addition of the proteasome inhibitor MG-132 generated significant signal for KLF15 protein in the presence of CVZ, which was canceled in the copresence of siRNA against GR or KLF15. Infection of adenovirus carrying flag-tagged KLF15 in cardiomyocytes induced exogenous KLF15 protein expression, which was again increased by MG132 treatment (Fig. 4D). These results further confirmed the role of glucocorticoids for cardiac KLF15 expression and suggested that KLF15 may be a labile and rapid turnover protein. Using this adenoviral system, we revealed that overexpression of KLF15 in cardiomyocytes significantly increased mRNA expression of BCAT2 and GLUT4 (Fig. 4E).

Next, we examined the role of glucocorticoids and KLF15 on amino acid metabolism in rat cardiomyocytes. Adenovirus-mediated overexpression of KLF15 decreased the concentrations of Val, Leu, and Ile (Fig. 5A), indicating that KLF15, most possibly via BCAT2 induction, may degrade branched-chain amino acid (BCAA). As previously reported (41), treatment of cardiomyocytes with CVZ upregulated mRNA expression of glutamine synthase, which catalyses condensation of Glu and ammonia to form Gln (Supplemental Table 1, and also see Ref. 21) and increased Gln with a reciprocal decrease in Glu (Fig. 5B). However, this alteration in the concentrations of Glu to Gln was not affected by siRNA-mediated knockdown of KLF15 (Fig. 5B). In clear contrast, the concentrations of Val, Leu, and Ile were decreased after treatment with CVZ and affected by KLF15 knockdown (Fig. 5B). At this moment, the precise role of BCAA in cardiac physiology remains unknown. In peripheral tissues, BCAA is shown to play an important role in multiple metabolic processes, including regulation of insulin sensitivity, protein synthesis, and energy production and expenditure (18, 23, 43). Further study, therefore, might clarify an as yet unidentified physiological role of glucocorticoids via alteration in amino acid composition in the heart.

**Glucocorticoids enhance prostaglandin biosynthesis via GR.** GO analysis also revealed the role of glucocorticoids in lipid metabolism in rat cardiomyocytes (Supplemental Table 2). Notably, it was striking that glucocorticoid-GR signaling promotes gene expression of the enzymes involved in the prostaglandin biosynthesis, including PLA2 and COX-2 in cardiomyocytes (Supplemental Tables 1 and 2), since this issue appears to be contradictory to the current knowledge that glucocorticoids elicit their anti-inflammatory properties via suppression of inflammatory induction of PLA2 and COXs and subsequent synthesis of proinflammatory prostaglandins (37). However, we confirmed our microarray data in qRT-PCR. As shown in Fig. 6, A and B, CVZ and COR significantly induced mRNA expression of these genes in a dose-dependent fashion, and these gene expressions were efficiently canceled by the GR antagonist RU-486. Moreover, introduction of siRNA against GR diminished the glucocorticoid-mediated upregulation of mRNA expression of PLA2 and COX-2 (Fig. 6C). We also confirmed this issue at protein levels in Western blot analysis as well. COX-2 protein expression was enhanced by 10.5- and 2.8-fold after treatment with CVZ and COR, respectively. On the other hand, other steroid hormones, including ALD, estradiol, and progesterone, did not significantly induce COX-2 protein expression (Fig. 6D). This glucocorticoid-mediated upregulation of COX-2 protein expression was almost comparable to that after treatment with IL-1β and lipopolysaccharide and was not observed in cardiac fibroblasts (Fig. 6D and data not shown). Glucocorticoid also induced mRNA expression of COX-1 and prostaglandin D2 synthase by a lesser degree compared with that of COX-2 and PLA2 (Supplemental Table 1 and data not shown).

During the preparation of this manuscript, it was reported that COX-2 are induced by glucocorticoids in cultured rat cardiomyocytes (46). Our present work strongly indicates that glucocorticoid triggers the production of a certain class of prostaglandins/eicosanoids via induction of mRNA expression of these enzymes. Recently, it was shown that both COX-1 and COX-2 are expressed in the myocardium and that selective COX inhibitor caused an incomplete inhibition of prostaglandin E2 (PGE2) production from heart muscle (47), indicating that both COX isoforms are enzymatically active and contribute to PGE2 generation in the myocardium. Using cultures of
rat neonatal ventricular myocytes, Mendez and Lapointe (31) demonstrated an induction of COX-2 in vitro. Liu and coworkers (29) found a constitutive expression of both COX isoforms in rat hearts, which was enhanced by lipopolysaccharide infused in vivo. The biological function of COX-2 in cardiomyocytes might be of major clinical concern, since the pharmacological role of COX-2 inhibitor still remains to be clarified (7). Further study is now ongoing to identify which eicosanoid products are mainly generated in cardiomyocytes under exposure to excess glucocorticoids and to clarify the role of such products in cardiac physiology.

In conclusion, our ligand-based approach involving CVZ and RU-486 as well as COR and ALD appears to be powerful to comprehensively identify target genes of the glucocorticoid-GR system. We think that such an approach could be applicable to an in vivo model as well as cultured cells. Because GR-MR redundancy is hazardous for identification of physiological function of corticosteroids in nonepithelial tissues that express both receptors but not 11β-HSD2, our approach may be desired for such purposes.

Recent basic and clinical studies have highlighted the role of glucocorticoid signaling in cardiac physiology and pathophysiology. Our ligand-based microarray analyses have clearly demonstrated that glucocorticoid-GR signaling may play various roles via alteration in the gene expression program and control complexed metabolic milieu in cardiomyocytes. Because ALD did not significantly contribute to expression of a majority of those genes that were induced via GR, we may strengthen that not MR but rather GR signaling should have important roles for maintenance of cardiomyocyte function, at least in the neonatal stage. Moreover, it is of particular interest that glucocorticoids are shown to be involved in amino acid catabolism and prostaglandin biosynthesis in the heart. In any case, further studies, therefore, should be performed to clarify how these corticosteroid-receptor systems coordinately regulate the gene expression program in concert with endocrine systems and contribute to maintenance of cardiac function.

ACKNOWLEDGMENTS

We thank Takako Maruyama for excellent technical assistance, members of the Morimoto Laboratory for help and discussion, and Drs. S. Nishitani, N. Ono, and K. Takehana (Pharmaceutical Research Laboratories, Ajinomoto) for excellent technical assistance in amino acid analysis.

Fig. 6. Glucocorticoids regulate prostaglandin metabolism in the cardiomyocytes. A: time course of mRNA expression of Ptgs2 (cyclooxygenase-2; COX-2) and Pla2g4a (cytoplasmic phospholipase A2; PLA2) in neonatal rat cardiomyocytes. The cells were cultured in the presence of each ligand for the indicated time periods, and mRNA expression of COX-2 and PLA2 was determined with qRT-PCR as described in MATERIALS AND METHODS. mRNA expression levels were normalized to Gapdh mRNA, and relative expression levels to the vehicle-treated samples at each time point are presented as fold (means ± SE of 3 independent experiments are shown). RU, RU-486. *P < 0.05 vs. cells treated with vehicle. B: concentration-dependent regulation of Ptgs2 and Pla2g4a gene expression by glucocorticoids. Total RNA was isolated from neonatal rat cardiomyocytes after treatment with the indicated concentrations of CVZ (circles) or COR (squares) for 3 h and analyzed in qRT-PCR as described in MATERIALS AND METHODS. *P < 0.05 vs. cells treated with vehicle. C: effect of GR knockdown on glucocorticoid-dependent induction of mRNA expression of COX-2 and PLA2. Neonatal rat cardiomyocytes were transfected with siRNA oligonucleotide for GR or control siRNA as indicated and cultured for 24 h. Next, the cells were treated with vehicle or 100 nM CVZ and COR for 12 h. Total RNA was prepared and analyzed with qRT-PCR. *P < 0.05 vs. vehicle-treated cells. D: effects of glucocorticoids on COX-2 protein expression in neonatal rat cardiomyocytes. Rat cardiomyocytes (left) or cardiac fibroblasts (right) were treated with vehicle or 10 ng/ml interleukin (IL)-1β, 100 ng/ml lipopolysaccharide (LPS), 100 nM each of estradiol (E2), progesterone (PROG), CVZ, COR, ALD, or 10 μM RU-486 (RU). Whole cell extracts were prepared, and 10 μg of protein were separated in SDS-PAGE. Protein expression of COX-2 and α-actinin was analyzed in Western blot as described in MATERIALS AND METHODS. Experiments were repeated 3 times with almost identical results, and representative results are shown.
GRANTS

This work was partly supported by a grant for Research on Intractable Diseases from the Japanese Ministry of Health, Labour and Welfare, KAKENHI (17GS0419, 20790657), and Nakatomi Foundation.

REFERENCES


