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 transfection reagent (Invitrogen) and added to the culture according to the manufacturer's protocol. 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 replaced 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 x *g*. Luciferase enzyme activity was determined using Luciferase Assay System (Promega) and a luminometer (Promega) according to the manufacturer's protocol. Relative light units (RLU) 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 RU486. 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 twice; first experiment (vehicle, COR, ALD, or CVZ treatment),

second experiment (vehicle, COR, ALD, CVZ, RU486, COR+RU486, ALD+RU486, or CVZ+RU486 treatment). Using pooled RNA samples, preparation of the labeled cRNA and microarray hybridization were performed by the Bio Matrix Research (Nagareyama, Japan) as follows. Isolated total RNA were amplified and labeled as described in the GeneChip Expression Analysis Technical Manual (Affymetrix, Santa Clara, CA). First, total RNA (1 μg) was converted into double-stranded cDNA using the One-Cycle cDNA Synthesis Kit (Affymetrix). Double-stranded cDNA was 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 a UV spectrophotometer. Next, cRNA (15 μg) was fragmented at 94°C in the presence of a fragmentation buffer (Affymetrix). Fifteen micrograms of the cRNA was hybridized to 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 hr at 45°C, then automatically washed and stained with 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 of 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 call 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, Ingenuity Systems Inc., 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 terms were performed by using ExPlain™ (BIOBASE, Wolfenbüttel, Germany, www.biobase.de). The data discussed in this publication have been deposited in National Center for Biotechnology Information (NCBI) 's Gene Expression Omnibus (GEO, (11)) and are accessible through GEO Series accession number GSE12752 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE12752). Referees can login by using User ID 'noritadayoshikawa' and Password 'nyoshikawa'.

Real-time quantitative RT-PCR (qRT-PCR)

Total RNA from primary cultures of cardiomyocytes was reverse-transcribed with oligo-dT primers using SuperScript™III First-Strand Synthesis System for RT-PCR (Invitrogen). 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 standard curves generated for each gene and mRNA for *Gapdh* was used as an internal control. The primer sequences used in this study are,

Gapdh: 5'-agccacatcgctcagaca-3' and 5'-gcccaatacgaccaaatcc-3'

Klf15: 5'- ctgcagcaagatgtacaccaa-3' and 5'- tcatctgagcgtgaaaacctc-3'

Bcat2: 5'- gtcggtgactgcaagttgg-3' and 5'- cctttcttctgggcttcttg-3'

Slc2a4 (glucose transporter 4, GLUT4): 5'- tgcagtgcctgagtcttcttt-3' and 5'-ccagtcactcgctgctga-3'

Foxo1a: 5'-tcaggctaggagttagtgagca-3' and 5'-ggggtgaagggcatcttt-3'

Fbxo32 (atrogin-1): 5'-cactctacactggcaacagca-3' and 5'-ggtgatcgtgagacctttgaa-3'

Gdf8 (myostatin): 5'-tgggcatgatcttgctgtaa-3' and 5'-tgttactttgacttctaaaaagggatt-3'

Sgk1: 5'-ctcctatgcatgcaaacacc-3' and 5'-tttgttgagagggacttggag-3'

Nppb (BNP): 5'-gtcagtcgcttgggctgt-3' and 5'-cagagctggggaaagaagag-3'

Ptgs2 (cyclooxygenase-2, COX-2): 5'-accaacgctgccacaact-3' and 5'-gcccaatacgaccaaatcc-3'

Pla2g4a (cytoplasmic phospholipase A2, PLA2): 5'-tctcatttaactctgggaactgc-3' and 5'-cagctgcaggaattctcacac-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. Then, the medium was replaced to fresh OPTI-MEM and the cells were further cultured with or without CVZ for 24 h. The cells were washed three times with PBS and lysed in 1ml of ice cold methanol for 5 min, except for the dish with same protocols for counting the number of cells. Cellular lysates and recovery efficiency control Phe-d5 were collected into 15 ml tubes, one ml of chloroform was added to the lysates, and the mixtures were briefly voltexed. The mixtures were centrifuged at 1000 x *g*, 4°C for 5 min and the supernatants were transferred into new 15 ml tubes. This chloroform precipitation method was again repeated and the supernatants were concentrated and dried with AES2010 SpeedVac system (Savant Instruments, Holbrook, NY), and redisolved 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 the means ± SEM 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 to 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 COS7 cells. After treatment with 100 nM of COR or ALD, both rat GR and MR translocated into 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 a GR antagonist RU486 shut down GR-dependent GRE-luciferase reporter gene activation by either CVZ, COR, or ALD, however, RU486 did not repress ALD or COR-inducible MR-dependent reporter gene activation (Fig. 1B and 1D). We, therefore, concluded that CVZ and RU486 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 such set of genes expression of that is influenced by GR, we analyzed gene expression changes after exposure of cells to COR, ALD, and CVZ in the absence or presence of RU486. Since 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 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 the Supplemental Table 1). Among 12,379 genes, 7,351, 7478, 7507, 7803, 7863, and 7,845 genes were considered to be relevant for further analysis for CVZ, COR, ALD, CVZ+RU486, COR+RU486, and ALD+RU486-treated cells, respectively (See Materials and Methods for details). 400 genes were significantly induced and 57 genes were repressed after treatment with either CVZ, COR, or ALD (Table 1). For classification, 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), 87 (categories 3, 5, 6 and 7) genes, respectively, with significant overlap between each (Fig. 2A). RU486-sensitivity of the genes in categories 1, 4, and 7 was 91.1% (for CVZ), 95.1% (for CVZ) and 79.6% (for COR), and 94.6% (for CVZ) and 75% (for COR), respectively (Table 1). We, therefore, may indicate that expression of 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 FKBP5, 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 by COR but not by 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. RU486-sensitivity of those 33 genes belonging to categories 2 and 5 were 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 RU486-sensitivity was equivocal (Supplemental Table 1), and it appears that the mode of their gene expression might be distinct from that of canonical GR target genes. Interestingly, RU486-sensitivity of the genes in category 7 appeared to be lower in ALD-induced cases than in CVZ- or COR-induced cases (Supplemental Table 1), suggesting that RU486-sensitivity of not all but some GR target genes may be influenced by ligand context.

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 ALD-induced gene set. Moreover, fold inducibility of majority of ALD-induced genes appeared to be marginal (Supplemental Table 1) and RU486-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.

Total number of down-regulated genes (n=57) was smaller than that of up-regulated 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, RU486 is known to have similar transrepressive effect when compared with agonistic glucocorticoids including CVZ and COR (17). 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.

Gene Ontology (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, Thompson et al. 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 (32). 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 RU486 (Supplemental Table 1). It, therefore, may be concluded that FKBP5 gene expression is driven by glucocorticoid-GR axis. Since FKBP5 is shown to be contained in GR chaperon complex with hsp90, this result may indicate that ultrashort feedback loop of GR operates in cardiomyocytes (4). Glucocorticoids have known to induce myocardial hypertrophy *in vivo*, however, and the effects of glucocorticoids on the cell size of cardiomyocytes are still controversial *in vitro* (9, 14, 26, 51). Indeed, several reports have suggested that treatment of cardiomyocytes with corticosterone 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 brain natriuretic peptide (BNP) (Supplemental 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 regulator of muscle mass via 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 intra-cellular factors, *e.g.*, hypertension and metabolic status. Indeed, it has been reported that glucocorticoid-induced cardiac enlargement of rat heart was transient and extension of treatment duration with 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 (Ppargc1a), 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 RU486, and their mRNA expression is also considered to be transcriptionally regulated by GR (Supplemental Table 2). Since not all but many of them are known to be involved in various metabolic processes (10), our results may indicate that glucocorticoid-GR modulates complex metabolic milieus via cascade of regulation of gene expression in the heart.

Glucocorticoid-mediated amino acid catabolism via KLF15 pathway

In the present study, Ingenuity Pathway Analysis returned the highest score to the gene network involving KLF15 and correlating with cardiovascular system development and function, amino acid metabolism, and small molecular biochemistry (Supplemental Table 3). KLFs are a subclass of the zinc finger family of DNA-binding transcription factors and recent

studies have revealed physiological importance of several members of KLF family in the heart and vessels (3). Especially, KLF15 was recently reported to be an inhibitor of cardiac hypertrophy (12). KLF15 is also considered to be involved in amino acid catabolism to induce branched-chain aminotransferase 2 (BCAT2) gene expression of which is a 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. 4A and 4B). 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 glucose transporter 4 (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 lag time of ~3-6 h after apparent induction of KLF15 mRNA in cardiomyocytes (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 glucocorticoid-GR-KLF15 pathway for BCAT2 gene expression.

Next, we further addressed the role of GR-dependent KLF15 induction in the cardiomyocytes. In Western blot analysis, KLF15 protein band was not detected after treatment of cardiomyocytes with CVZ alone. However, addition of the proteasome inhibitor MG132 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 into 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 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 reciprocal decrease in Gln (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 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 phospholipase A2 (PLA2) and cyclooxygenase-2 (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. 6A and 6B, CVZ and COR significantly induced mRNA expression of these genes in a dose-dependent fashion and these gene expressions were efficiently canceled by GR antagonist RU486. Moreover, introduction of siRNA against GR diminished these 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 with lesser degree when 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 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 (PGE_2) production from heart muscle (47),

indicating that both COX isoforms are enzymatically active and contribute to PGE_2 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 the cardiomyocytes might be of major clinical concern, since 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.

Concluding remarks

Our ligand-based approach involving CVZ and RU486 as well as COR and ALD appears to be powerful to comprehensively identify target genes of glucocorticoid-GR system. We think that such approach could be applicable to in vivo model as well as cultured cells. Since GR-MR redundancy is hazardous for identification of physiological function of corticosteroids in non-epithelial tissues which express both receptor but not 11β-HSD2, our approach may be deserved for such purposes.

Recent basic and clinical studies have highlighted the role of corticosteroid 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 gene expression program and control complexed metabolic milieus in cardiomyocytes. Since ALD did not significantly contribute to expression of 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 cardiomyocytes function, at least in 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 coordinatedly regulate gene expression program in concert with endocrine systems and contribute to maintenance of cardiac function.

Acknowledgements

This work was partly supported by the grant for Research on Intractable Diseases from Japanese Ministry of Health, Labour and Welfare, KAKENHI (17GS0419, 20790657), and Nakatomi Foundation. We thank Ms. Takako Maruyama for excellent technical assistance, members of Morimoto Lab for help and discussion, and Drs. S. Nishitani, N. Ono, and K. Takehana (Pharmaceutical Research Laboratories, Ajinomoto Co., Inc.) for excellent technical assistance in amino acid analysis.

References

1. **Almon RR, DuBois DC, Yao Z, Hoffman EP, Ghimbovschi S, and Jusko WJ.** Microarray analysis of the temporal response of skeletal muscle to methylprednisolone: comparative analysis of two dosing regimens. *Physiol Genomics* 30: 282-299, 2007.

2. **Arriza JL, Weinberger C, Cerelli G, Glaser TM, Handelin BL, Housman DE, and Evans RM.** Cloning of human mineralocorticoid receptor complementary DNA: structural and functional kinship with the glucocorticoid receptor. *Science* 237: 268-275, 1987.

3. **Atkins GB, and Jain MK.** Role of Kruppel-like transcription factors in endothelial biology. *Circulation research* 100: 1686-1695, 2007.

4. **Binder EB, Bradley RG, Liu W, Epstein MP, Deveau TC, Mercer KB, Tang Y, Gillespie CF, Heim CM, Nemeroff CB, Schwartz AC, Cubells JF, and Ressler KJ.** Association of FKBP5 polymorphisms and childhood abuse with risk of posttraumatic stress disorder symptoms in adults. *Jama* 299: 1291-1305, 2008.

5. **Brotman DJ, Girod JP, Garcia MJ, Patel JV, Gupta M, Posch A, Saunders S, Lip GY, Worley S, and Reddy S.** Effects of short-term glucocorticoids on cardiovascular biomarkers. *The Journal of clinical endocrinology and metabolism* 90: 3202-3208, 2005.

6. **Czerwinski SM, Kurowski TT, McKee EE, Zak R, and Hickson RC.** Myosin heavy chain turnover during cardiac mass changes by glucocorticoids. *J Appl Physiol* 70: 300-305, 1991.

7. **Davies NM, and Jamali F.** COX-2 selective inhibitors cardiac toxicity: getting to the heart of the matter. *J Pharm Pharm Sci* 7: 332-336, 2004.

8. **Davis JM, 3rd, Maradit Kremers H, Crowson CS, Nicola PJ, Ballman KV, Therneau TM, Roger VL, and Gabriel SE.** Glucocorticoids and cardiovascular events in rheumatoid arthritis: a population-based cohort study. *Arthritis Rheum* 56: 820-830, 2007.

9. **de Vries WB, van der Leij FR, Bakker JM, Kamphuis PJ, van Oosterhout MF, Schipper ME, Smid GB, Bartelds B, and van Bel F**. Alterations in adult rat heart after neonatal dexamethasone therapy. *Pediatr Res* 52: 900-906, 2002.

10. **Desvergne B, Michalik L, and Wahli W**. Transcriptional regulation of metabolism. *Physiol Rev* 86: 465-514, 2006.

11. **Edgar R, Domrachev M, and Lash AE**. Gene Expression Omnibus: NCBI gene expression and hybridization array data repository. *Nucleic Acids Res* 30: 207-210, 2002.

12. **Fisch S, Gray S, Heymans S, Haldar SM, Wang B, Pfister O, Cui L, Kumar A, Lin Z, Sen-Banerjee S, Das H, Petersen CA, Mende U, Burleigh BA, Zhu Y, Pinto YM, Liao R, and Jain MK**. Kruppel-like factor 15 is a regulator of cardiomyocyte hypertrophy. *Proc Natl Acad Sci U S A* 104: 7074-7079, 2007.

13. **Freerksen DL, and Hartzell CR**. Glucocorticoid stimulation of metabolism and glycerol-3-phosphate dehydrogenase activity in cultured heart cells. *J Cell Physiol* 126: 206-210, 1986.

14. **Gardner DG, Wu JP, LaPointe MC, Hedges BK, and Deschepper CF**. Expression of the gene for the atrial natriuretic peptide in cardiac myocytes in vitro. *Cardiovasc Drugs Ther* 2: 479-486, 1988.

15. **Gray S, Feinberg MW, Hull S, Kuo CT, Watanabe M, Sen-Banerjee S, DePina A, Haspel R, and Jain MK**. The Kruppel-like factor KLF15 regulates the insulin-sensitive glucose transporter GLUT4. *J Biol Chem* 277: 34322-34328, 2002.

16. **Guder G, Bauersachs J, Frantz S, Weismann D, Allolio B, Ertl G, Angermann CE, and Stork S**. Complementary and incremental mortality risk prediction by cortisol and aldosterone in chronic heart failure. *Circulation* 115: 1754-1761, 2007.

17. **Honer C, Nam K, Fink C, Marshall P, Ksander G, Chatelain RE, Cornell W, Steele R, Schweitzer R, and Schumacher C**. Glucocorticoid receptor antagonism by

cyproterone acetate and RU486. *Mol Pharmacol* 63: 1012-1020, 2003.

18. **Hutson SM, Sweatt AJ, and Lanoue KF**. Branched-chain [corrected] amino acid metabolism: implications for establishing safe intakes. *J Nutr* 135: 1557S-1564S, 2005.

19. **Iwatani S, Van Dien S, Shimbo K, Kubota K, Kageyama N, Iwahata D, Miyano H, Hirayama K, Usuda Y, Shimizu K, and Matsui K**. Determination of metabolic flux changes during fed-batch cultivation from measurements of intracellular amino acids by LC-MS/MS. *Journal of biotechnology* 128: 93-111, 2007.

20. **James CG, Ulici V, Tuckermann J, Underhill TM, and Beier F**. Expression profiling of Dexamethasone-treated primary chondrocytes identifies targets of glucocorticoid signalling in endochondral bone development. *BMC Genomics* 8: 205, 2007.

21. **Kanda F, Okuda S, Matsushita T, Takatani K, Kimura KI, and Chihara K**. Steroid myopathy: pathogenesis and effects of growth hormone and insulin-like growth factor-I administration. *Hormone research* 56 Suppl 1: 24-28, 2001.

22. **Kniss DA, and Burry RW**. Glucocorticoid hormones inhibit DNA synthesis in glial cells cultured in chemically defined medium. *Exp Cell Res* 161: 29-40, 1985.

23. **Kodde IF, van der Stok J, Smolenski RT, and de Jong JW**. Metabolic and genetic regulation of cardiac energy substrate preference. *Comparative biochemistry and physiology* 146: 26-39, 2007.

24. **Komamura K, Shirotani-Ikejima H, Tatsumi R, Tsujita-Kuroda Y, Kitakaze M, Miyatake K, Sunagawa K, and Miyata T**. Differential gene expression in the rat skeletal and heart muscle in glucocorticoid-induced myopathy: analysis by microarray. *Cardiovasc Drugs Ther* 17: 303-310, 2003.

25. **Kurowski TT, and Czerwinski SM**. Glucocorticoid modulation of cardiac mass and protein. *Med Sci Sports Exerc* 22: 312-315, 1990.

26. **La Mear NS, MacGilvray SS, and Myers TF**. Dexamethasone-induced myocardial

hypertrophy in neonatal rats. *Biol Neonate* 72: 175-180, 1997.

27. **Lefer AM**. Influence of corticosteroids on mechanical performance of isolated rat papillary muscles. *The American journal of physiology* 214: 518-524, 1968.

28. **Lister K, Autelitano DJ, Jenkins A, Hannan RD, and Sheppard KE**. Cross talk between corticosteroids and alpha-adrenergic signalling augments cardiomyocyte hypertrophy: a possible role for SGK1. *Cardiovasc Res* 70: 555-565, 2006.

29. **Liu SF, Newton R, Evans TW, and Barnes PJ**. Differential regulation of cyclo-oxygenase-1 and cyclo-oxygenase-2 gene expression by lipopolysaccharide treatment in vivo in the rat. *Clin Sci (Lond)* 90: 301-306, 1996.

30. **Menconi M, Fareed M, O'Neal P, Poylin V, Wei W, and Hasselgren PO**. Role of glucocorticoids in the molecular regulation of muscle wasting. *Crit Care Med* 35: S602-608, 2007.

31. **Mendez M, and LaPointe MC**. Trophic effects of the cyclooxygenase-2 product prostaglandin E(2) in cardiac myocytes. *Hypertension* 39: 382-388, 2002.

32. **Miller AL, Webb MS, and Thompson EB**. Comparison of two structurally diverse glucocorticoid receptor agonists: cortivazol selectively regulates a distinct set of genes separate from dexamethasone in CEM cells. *Steroids* 72: 673-681, 2007.

33. **Narayanan N, Yang C, and Xu A**. Dexamethasone treatment improves sarcoplasmic reticulum function and contractile performance in aged myocardium. *Mol Cell Biochem* 266: 31-36, 2004.

34. **Pazirandeh A, Xue Y, Prestegaard T, Jondal M, and Okret S**. Effects of altered glucocorticoid sensitivity in the T cell lineage on thymocyte and T cell homeostasis. *Faseb J* 16: 727-729, 2002.

35. **Penefsky ZJ, and Kahn M**. Inotropic effects of dexamethasone in mammalian heart muscle. *Eur J Pharmacol* 15: 259-266, 1971.

36. **Phuc Le P, Friedman JR, Schug J, Brestelli JE, Parker JB, Bochkis IM, and Kaestner KH**. Glucocorticoid receptor-dependent gene regulatory networks. *PLoS Genet* 1: e16, 2005.

37. **Rhen T, and Cidlowski JA**. Antiinflammatory action of glucocorticoids--new mechanisms for old drugs. *N Engl J Med* 353: 1711-1723, 2005.

38. **Rupprecht R, Arriza JL, Spengler D, Reul JM, Evans RM, Holsboer F, and Damm K**. Transactivation and synergistic properties of the mineralocorticoid receptor: relationship to the glucocorticoid receptor. *Molecular endocrinology* 7: 597-603, 1993.

39. **Sainte-Marie Y, Nguyen Dinh Cat A, Perrier R, Mangin L, Soukaseum C, Peuchmaur M, Tronche F, Farman N, Escoubet B, Benitah JP, and Jaisser F**. Conditional glucocorticoid receptor expression in the heart induces atrio-ventricular block. *Faseb J* 21: 3133-3141, 2007.

40. **Sano M, Tokudome S, Shimizu N, Yoshikawa N, Ogawa C, Shirakawa K, Endo J, Katayama T, Yuasa S, Ieda M, Makino S, Hattori F, Tanaka H, and Fukuda K**. Intramolecular control of protein stability, subnuclear compartmentalization, and coactivator function of peroxisome proliferator-activated receptor gamma coactivator 1alpha. *J Biol Chem* 282: 25970-25980, 2007.

41. **Schlechte JA, and Schmidt TJ**. Use of [3H]cortivazol to characterize glucocorticoid receptors in a dexamethasone-resistant human leukemic cell line. *The Journal of clinical endocrinology and metabolism* 64: 441-446, 1987.

42. **Schulz R, Nava E, and Moncada S**. Induction and potential biological relevance of a Ca(2+)-independent nitric oxide synthase in the myocardium. *Br J Pharmacol* 105: 575-580, 1992.

43. **She P, Reid TM, Bronson SK, Vary TC, Hajnal A, Lynch CJ, and Hutson SM**. Disruption of BCATm in mice leads to increased energy expenditure associated with the