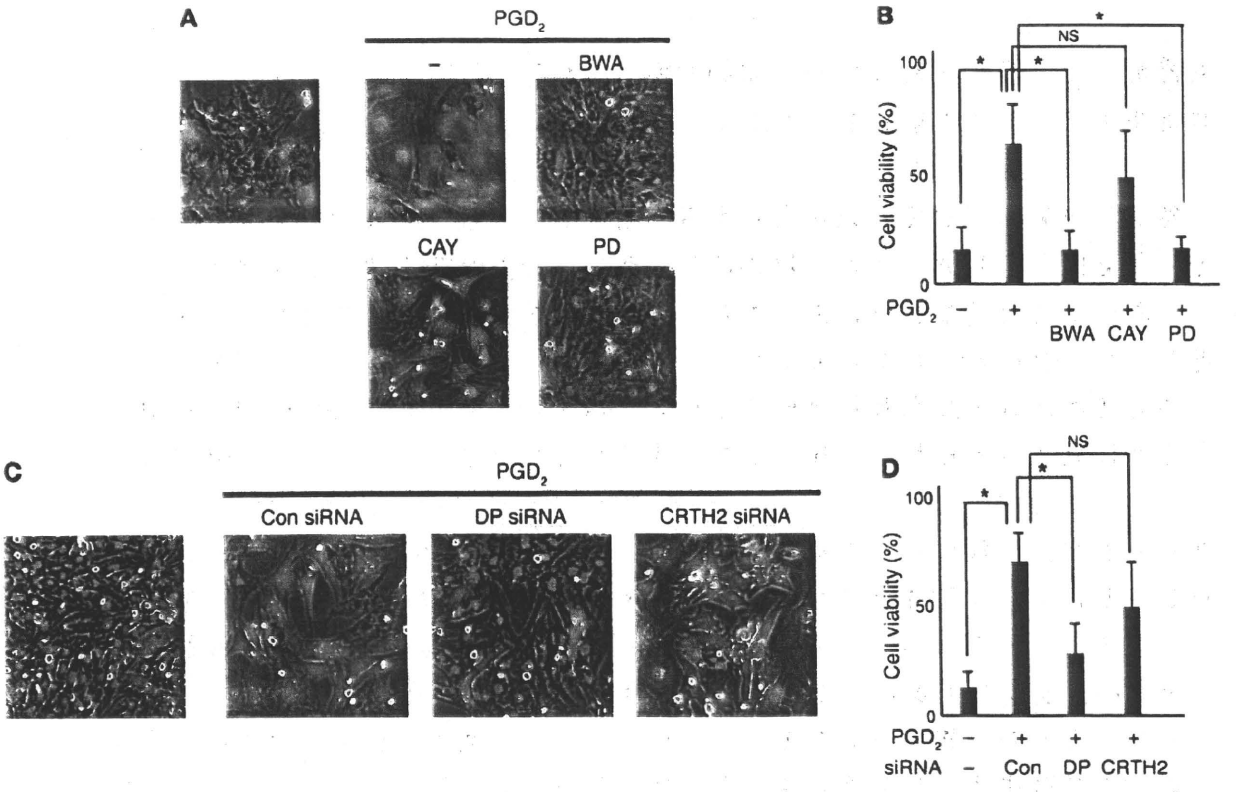

Figure 10

PGD_2 protects against cardiomyocyte death induced by anoxia/reoxygenation injury via the DP receptor and ERK1/2 signaling. (A and B) Neonatal rat cardiomyocytes were stimulated with 30 nM PGD_2 in the presence or absence of 10 μM BWA868C, 10 μM CAY10471, or 20 μM PD98059 (PD) and then subjected to glucose-free anoxia followed by reoxygenation. (A) Dead cells (red nuclei) and viable cells (green) were determined as described in Methods. Scale bars: 100 μm. (B) Viable cells were quantified by counting 100 cells in 5 independent experiments ($n = 5$). (C and D) Neonatal rat cardiomyocytes were transfected with control-, DP-, or CRTH2-specific siRNA. Reductions in the expression levels of the target genes were confirmed by Q-PCR (see Supplemental Figure 5). Cells were treated with 30 nM PGD_2 and then subjected to glucose-free anoxia followed by reoxygenation. (C) Dead cells (red nuclei) and viable cells (green) were determined as described in Methods. Scale bars: 100 μm. (D) Viable cells were quantified by counting 100 cells in 5 independent experiments ($n = 5$). *$P < 0.05$; Student's t test.

itation, GR-selective agonists, such as DEX and betamethasone, may have clinical advantages over nonselective GR agonists (e.g., prednisolone and cortisol) in limiting infarct size and improving mortality after myocardial infarction.

Methods

Animals. All animal experiments were reviewed and approved by the Institutional Animal Care and Use Committee at the Keio University School of Medicine. L-PGDS–knockout mice were generated as previously described (39).

Gene expression. Neonatal ventricular myocytes from 1- to 2-day-old Sprague-Dawley rats were subjected to Percoll gradient centrifugation and differential plating in order to enrich for cardiac myocytes and deplete nonmyocyte populations (40). Total RNA was isolated and hybridized to the GeneChip Rat Genome 230 2.0 Array (Affymetrix), composed of 31,042 probe sets representing approximately 28,000 rat genes. Q-PCR was performed using the ABI Prism 7700 sequence detection system (Applied Biosystems). Predesigned gene-specific primer and probe sets (TaqMan Gene Expression Assays) were used. The 18s ribosomal RNA was amplified as an internal control.

Western blotting. Nuclear extracts were prepared as previously described (41). Rabbit polyclonal antibodies against GR and goat polyclonal antibodies against COX2 were purchased from Santa Cruz Biotechnology. Rabbit polyclonal antibodies directed against COX-1 were purchased from Alexis, and rabbit polyclonal antibodies directed against L-PGDS, mPGES1, mPGES2, cytosolic PGE synthase, DP, and CRTH2 were purchased from Cayman Chemical. Protein expression was visualized using horseradish peroxidase–conjugated secondary antibodies and enhanced chemiluminescence (Amersham Biosciences) and was detected using the LAS-3000 luminoimage analyzer (Fujifilm).

ELISA. Neonatal rat cardiomyocytes were treated with 100 nM CVZ for 24 hours. The levels of PGD_2, PGE_2, and 6-keto-$PGF_{1\alpha}$ in the culture media were measured using a commercial ELISA kit (Cayman Chemical) according to the manufacturer's instructions.

Oxidized phospholipid analysis by LC-MS/MS. The frozen hearts were homogenized in methanol that contained 2 internal standards (LTB4-d4 and 17:0-LPC), and oxidative fatty acids and oxidative phospholipids were extracted using solid-phase extraction. LC-MS/MS analysis was performed using the 4000 Q-TRAP quadrupole linear ion-trap hybrid mass spectrometer (Applied Biosystems/MDS Sciex) with the ACQUITY Ultra Performance LC (Waters). Specific detection was performed by multiple reaction monitoring (18).

Glucose-free hypoxia and reoxygenation. An anaerobic jar that contained an Anaero Pack (Mitsubishi Gas Chemical) was used to expose the cells to hypoxic stress (42). The medium used to grow the cardiomyocytes was replaced with glucose-free DMEM before the cells were exposed to hypoxic stress. After 5 hours of exposure of hypoxia, the medium was replaced

with 10% FBS-containing DMEM (reoxygenation medium). Cell viability was determined by the LIVE/DEAD Viability/Cytotoxicity Assay Kit (Invitrogen) based on the simultaneous determination of live and dead cells with the calcein AM and ethidium homodimer-1 probes, which are specific for intracellular esterase activity and membrane integrity, respectively. Fluorescence imaging of the cells was performed with a fluorescence microscope (BZ-9000; Keyence): live cells were labeled green, whereas the nuclei of dead cells were labeled red.

siRNA oligonucleotides and transfection. siRNA oligonucleotides against the rat *GR*, *Ptgs2*, and *Ptgds* genes, as well as control siRNA, were purchased from Ambion. Transfection of these siRNA oligonucleotides was performed using the Lipofectamine RNAiMAX Reagent (Invitrogen).

Langendorff perfusion of the heart. Hearts were excised rapidly from heparinized mice, perfused with modified Krebs-Henseleit buffer (120 mmol/l NaCl, 25 mmol/l $NaHCO_3$, 5.9 mmol/l KCl, 1.2 mmol/l $MgSO_4$, 1.75 mmol/l $CaCl_2$, and 10 mmol/l glucose), and gassed with 95% O_2 and 5% CO_2 at 37°C according to the Langendorff procedure. Coronary perfusion pressure was maintained at 90 mmHg. A plastic catheter with a polyethylene balloon was inserted into the LV through the left atrium. Before the induction of ischemia, the LV end-diastolic pressure (LVEDP) was adjusted to 10 mmHg by filling the balloon with water. Indices of LV function (LV systolic pressure; LVEDP; LVDP, calculated as the difference between LVSP and LVEDP; $+dP/dt$; and $-dP/dt$) were recorded as described previously (43). Rate-pressure product was calculated as the product of LV systolic pressure and heart rate. Total LDH activity released into the perfusate was measured with a commercially available kit (Sigma-Aldrich).

In vivo myocardial ischemia/reperfusion model. Regional myocardial ischemia was induced by transient occlusion of the left anterior descending coronary artery. After 30 minutes of ischemia, the tube used for myocardial reperfusion we removed, and the thorax was closed with the suture intact. The suture around the coronary artery was retied 2 hours after reperfusion, and 2% Evans blue dye was injected into the LV cavity to delineate retrospectively the area at risk for myocardial infarction. The heart was removed, washed in PBS, and then sliced into sequential 1-mm-thick sections. The sections were stained with 2,3,5-triphenyltetrazolium chloride (TTC; 3%), and the infarct (white), noninfarct (red), nonischemic (blue), and at-risk areas (white and red) were measured.

Immunohistochemistry. Hearts were perfused from the apex with PBS, perfusion fixed with 4% paraformaldehyde in PBS, dissected, subsequently cryoprotected in sucrose solution at 4°C, embedded in OCT compound (Miles Scientific), and snap-frozen in liquid nitrogen. The fixed hearts were sectioned (8-μm thickness) using the CM3050S cryostat (Leica). For immunostaining, the sections were blocked in 5% BSA for 30 minutes at room temperature and then treated with anti-L-PGDS and anti-CD45 antibodies (BD Biosciences — Pharmingen) overnight at 4°C. Secondary antibodies conjugated to Alexa Fluor 546 or Alexa Fluor 488 (Invitrogen) were applied at 1:200 dilutions for 1 hour at 4°C. Nuclei were stained with TOTO-3 (Invitrogen) in a mounting medium. The slides were observed under a fluorescence microscope (Olympus BX-60).

Statistics. Values are presented as mean ± SEM. Statistical significance was evaluated using 2-tailed, unpaired Student's *t* tests for comparisons of 2 mean values. Multiple comparisons involving more than 3 groups were performed using ANOVA. A *P* value less than 0.05 was considered statistically significant.

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

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[1]*Division of Clinical Immunology, Advanced Clinical Research Center,* [2]*Research Hospital,* [3]*Laboratory of DNA Information Analysis, Human Genome Center, Institute of Medical Science, University of Tokyo, Tokyo;* [4]*Department of Regenerative Medicine and Advanced Cardiac Therapeutics,* [5]*Department of Biochemistry and Integrative Medical Biology, Keio University School of Medicine, Tokyo; and* [6]*Precursory Research for Embryonic Science and Technology, Japan Science and Technology Agency, Saitama, Japan*

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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 Ca^{2+} currents (51) and inhibits inducible nitric oxide synthase activity in rat cardiomyocytes (42). Moreover, DEX treatment enhances the development of contractile tension and increases contraction and relaxation velocities in cardiac muscle (35). The decrease in contractile force of rat papillary muscle induced by adrenalectomy is prevented by DEX treatment (27) by modulating membrane Ca^{2+} transport and K^+ channels (33, 35, 50, 51). Short-term treatment with DEX has been shown to decrease resting heart rate in healthy human volunteers (5). It, thus, 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 deleterious 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, several clinical 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 cortisol 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-

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coids because of the presence of 11β-hydroxysteroid dehydrogenase type II (11βHSD2), an enzyme that converts cortisol (human)/corticosterone (COR, rodents) into inactive metabolites. 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. Taking 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 glucocorticoid effects could be mediated by both GR and MR (48). The recent advent of microarray and other technologies has facilitated 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 electrophysiological phenotyping indicated that cardiac GR overexpression resulted in conduction defects, with high-degree atrio-ventricular 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, cortivazol (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-486 might be applied to define the role of GR in nonclassical ALD target tissues. In the present study, we performed microarray 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, lipopolysaccharide, 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-cyclooxygenase-2 (COX-2, sc-1747), and anti-KLF15 (sc-34827) antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-α-actinin (A7811) and anti-FLAG (F1804) antibodies were obtained from Sigma.

Plasmids, small-interfering RNA oligonucleotides, and recombinant 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 glucocorticoid response element (GRE)-driven reporter plasmid p2xGRE-LUC was described previously (52). Small-interfering RNA (siRNA) oligonucleotides 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 RSS340443) were purchased from Invitrogen (Carlsbad, CA). Recombinant adenoviruses encoding FLAG-tagged rat KLF15 and Cre-recombinase were generated by using the Adenovirus Cre/loxP-regulated Expression Vector Set (TaKaRa, Otsu, Japan) as per the manufacturer's instructions and as previously described (44). Recombinant adenoviruses prepared from 293 cells were purified with Virakit 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% CO_2. 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 1×10^5 cells/cm^2 on gelatin-coated dishes and grown in medium 199/DMEM (Invitrogen) supplemented with 10% FCS and antibiotics in a humidified atmosphere at 37°C with 5% CO_2. 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 temperature. 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 electrically transferred to a polyvinyl difluoride membrane (Millipore, Bedford, MA). Subsequently, immunoblotting was performed with anti-GR, anti-α-actinin, 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-actinin 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 transfection 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 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 *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 (vehicle, COR, ALD, or CVZ treatment) and second experiment (vehicle, COR, ALD, CVZ, RU-486, COR + RU-486, ALD + RU-486, or CVZ + RU-486 treatment). Using pooled RNA samples, preparation of the labeled cRNA and microarray hybridization were performed by 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 an ultraviolet spectrophotometer. Next, cRNA (15 μg) was fragmented at 94°C in the presence of a fragmentation buffer (Affymetrix). 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 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, 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 (BIOBASE, 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 System 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 standard curves generated for each gene, and mRNA for *glyceraldehyde-3-phosphate dehydrogense* (*Gapdh*) was used as an internal control. The primer sequences used in this study are as follows: *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* (*brain natriuretic peptide*, *BNP*): 5′-gtcagtcgcttgggctgt-3′ and 5′-cagagctggggaaagaagag-3′; *Ptgs2* (*COX-2*): 5′-accaacgctgccacaact-3′ and 5′-gcccaatacgaccaaatcc-3′; and *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. Next, 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 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 voltexed. 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 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 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. 1*A*, 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. 1*B*). 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. 1*A*) and transactivation potential (Fig. 1*B*) 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,

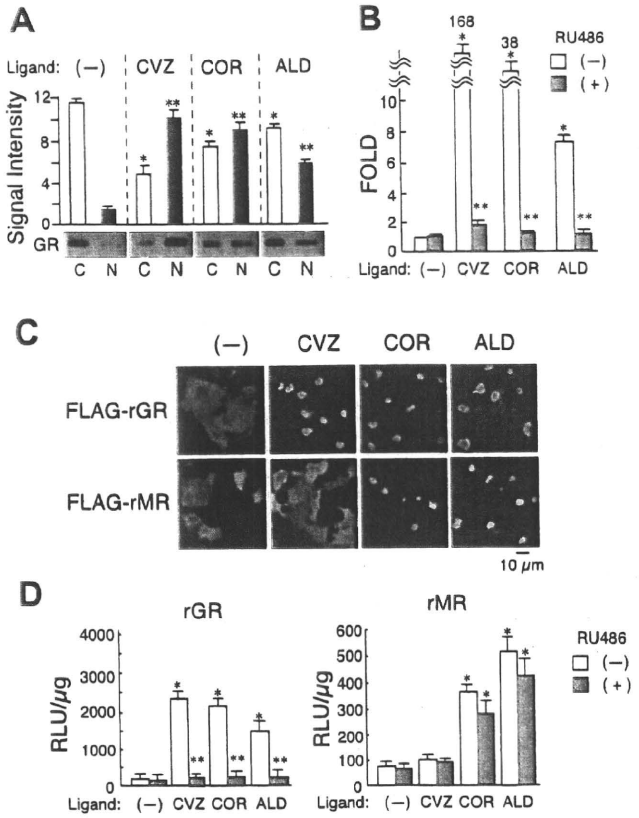

Fig. 1. Characterization of endogenous glucocorticoid receptor (GR) in rat cardiomyocytes and effects of synthetic and endogenous corticosteroids. *A*: subcellular localization of GR in neonatal rat cardiomyocytes. After treatment of the cardiomyocytes with or without 100 nM of each corticosteroid for 1 h as indicated, cytosolic extracts (C) and nuclear extracts (N) were prepared, Western immunoblotting for GR were performed, and signal intensity of the band for GR relative to that for α-actinin of each extract was quantified as described in MATERIALS AND METHODS. Means ± SE of 3 independent experiments and representative photographs are shown. $P < 0.05$ vs. cytosolic extract treated with vehicle (*) and vs. nuclear extracts treated with vehicle (**). CVZ, cortivazol; COR, corticosterone; ALD, aldosterone. *B*: effect of corticosteroids on glucocorticoid response element (GRE)-dependent reporter gene expression in neonatal rat cardiomyocytes. The cardiomyocytes were transfected with 2 μg of reporter plasmids p2xGRE-LUC and treated with 100 nM of each ligand as indicated in the presence (filled bars) or absence (open bars) of 10 μM RU-486 for 24 h. Results are expressed as relative expression levels to the vehicle-treated samples and means ± SE of 3 independent experiments are shown. $P < 0.05$ vs. cells treated with vehicle (*) and vs. RU-486 (−; **). *C*: ligand specificity of rat GR and mineralocorticoid receptor (MR) nuclear translocation. COS-7 cells expressing either FLAG-tagged rat GR or MR were cultured with or without 100 nM of each ligand as indicated for 2 h, and immunofluorescent analysis was performed as described in MATERIALS AND METHODS. Experiments were repeated 3 times with almost identical results, and representative results are shown. *D*: ligand specificity of rat GR and MR reporter gene assay. COS-7 cells were cotransfected with 2 μg of reporter plasmids p2xGRE-LUC and 100 ng of either p3xFLAG-rGR or p3xFLAG-rMR and were cultured with 100 nM of each ligand as indicated in the presence (filled bars) or absence (open bars) of 10 μM RU-486 for 24 h. Results are expressed as relative light units (RLU)/μg of protein in the extract, and means ± SE of 3 independent experiments are shown. $P < 0.05$ vs. cells treated with vehicle (*) and vs. RU-486 (−; **).

CVZ failed to promote nuclear translocation of not GR but MR (Fig. 1*C*). This issue is further supported by the luciferase assay in which CVZ again failed to induce MR-dependent reporter gene activation (Fig. 1*D*). It was also shown that the GR antagonist RU-486 shut down GR-dependent GRE-luciferase 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. 2*A*). RU-486 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 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 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 induced 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. 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

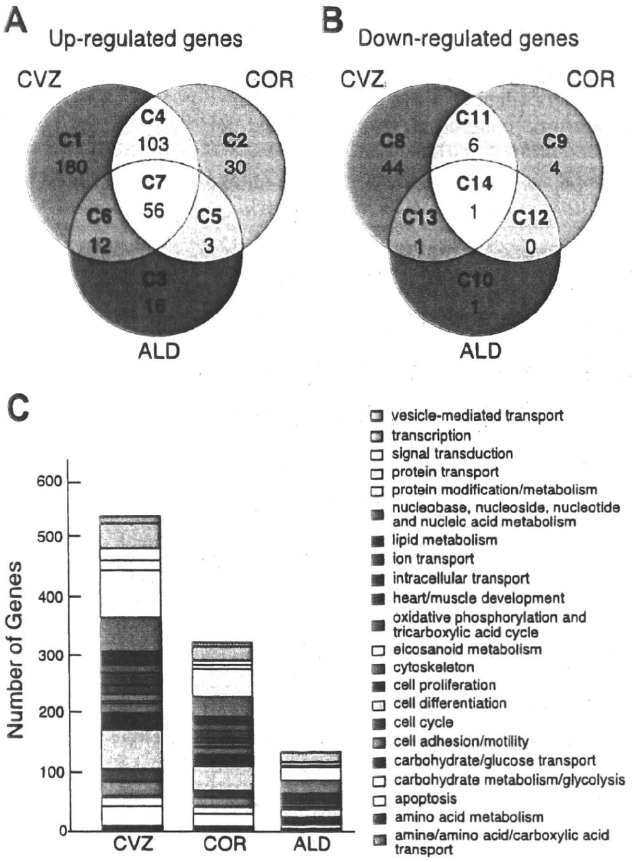

Fig. 2. Venn diagrams of corticosteroid-induced (*A*) or -repressed (*B*) genes and Gene Ontology analysis (*C*). *A* and *B*: venn diagrams. After isolation of total RNA from neonatal rat cardiomyocytes treated with either CVZ, COR, or ALD, with or without RU-486, DNA microarray analysis was performed, and data mining was done as described in MATERIALS AND METHODS. Venn diagrams illustrate the overlaps in CVZ, COR, and ALD target genes. The category numbers (C1–C14) and the number of upregulated (>1.5-fold vs. vehicle; *A*) and downregulated (<0.5-fold vs. vehicle; *B*) genes are depicted (See Table 1 and Supplemental Table 1 for details). *C*: Gene Ontology. The gene set induced by each corticosteroid was functionally classified according to Gene Ontology terms by using ExPlain software as described in MATERIALS AND METHODS, and results are shown.

Table 1); it appears that the mode of their gene expression might be distinct from that of canonical GR target genes. Interestingly, RU-486 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 RU-486 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 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. 2*B*). 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. 2*C*. 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. 2*C*). 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 GR chaperon complex with heat shosk protein-90, this result may indicate that the ultrashort feedback loop of GR operates in cardiomyocytes (4). Glucocorticoids have been known 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*

Category	Total	CVZ		COR		ALD	
		RU sensitive	RU insensitive	RU sensitive	RU insensitive	RU sensitive	RU insensitive
				No. of Upregulated Genes			
C1	180	164	16	0	0	0	0
C2	30	0	0	19	11	0	0
C3	16	0	0	0	0	8	8
C4	103	98	5	82	21	0	0
C5	3	0	0	1	2	2	1
C6	12	8	4	0	0	6	6
C7	56	53	3	42	14	23	33
Total	400	323	28	144	48	39	48
				No. of Downregulated Genes			
C8	44	38	6	0	0	0	0
C9	4	0	0	2	2	0	0
C10	1	0	0	0	0	0	1
C11	6	6	0	4	2	0	0
C12	0	0	0	0	0	0	0
C13	1	0	1	0	0	0	1
C14	1	0	1	0	1	0	1
Total	57	44	8	6	5	0	3

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, $([X + \text{RU-486}] - 1)/([X] - 1) < 0.5$; RU-insensitive, $([X + \text{RU-486}] - 1)/([X] - 1) > 0.5$ [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).

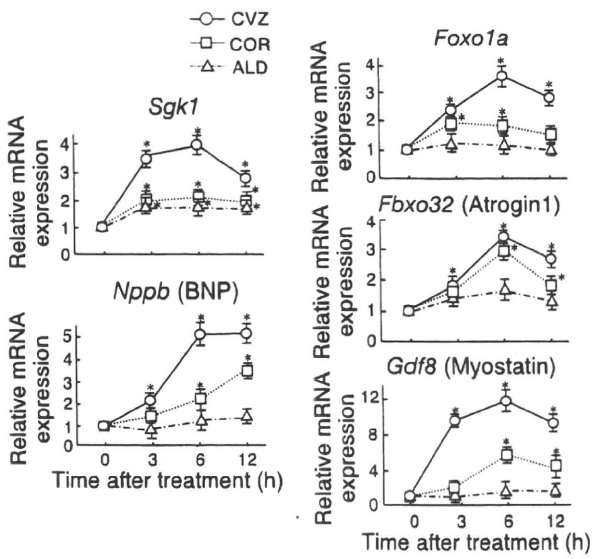

Fig. 3. Time course of mRNA expression of glucocorticoid-regulated genes in rat cardiomyocytes. Total RNA was isolated from neonatal rat cardiomyocytes after treatment with 100 nM CVZ (circles), COR (squares), or ALD (triangles) for the indicated time periods, and was analyzed in qRT-PCR as described 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.

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 milieus via cascade of regulation of gene expression in the heart.

Glucocorticoid-mediated amino acid catabolism via the 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 the physiological importance of several members of the KLF family in the heart and vessels (3). Especially, KLF15 was recently reported to be an inhibitor of cardiac hypertrophy (12). KLF15 is also con-

sidered 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. 4*C*), 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-

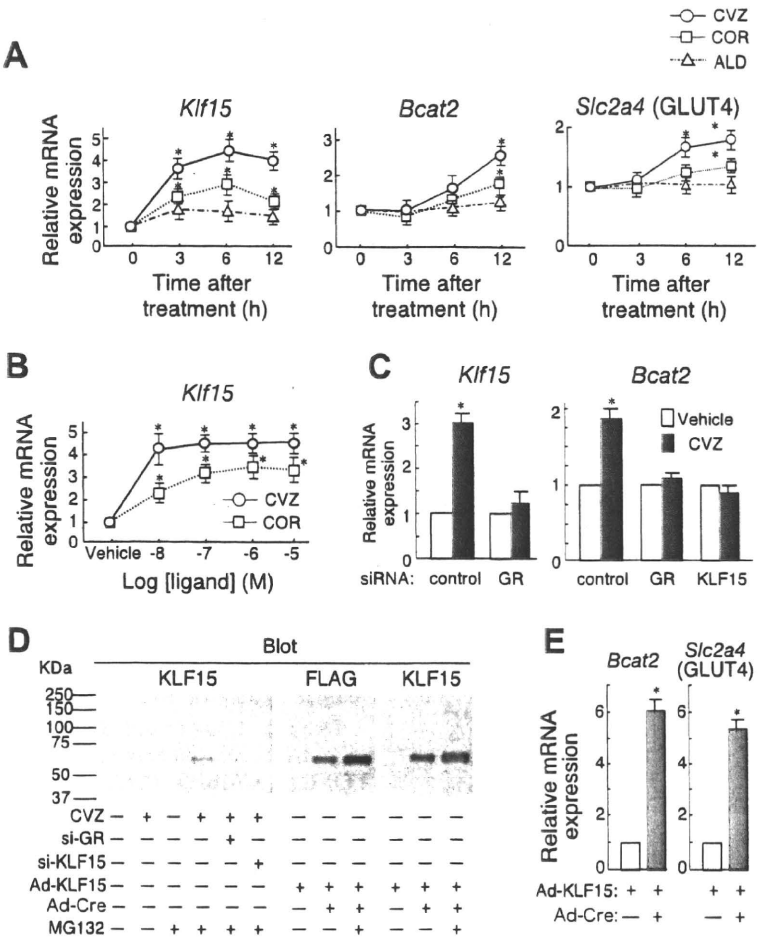

Fig. 4. KLF15 is a GR target gene involved in the amino acid catabolic pathway in rat cardiomyocytes. *A*: time course of mRNA expression of KLF15 and its target genes. Total RNA was isolated from neonatal rat cardiomyocytes after treatment with 100 nM of CVZ (circles), COR (squares), or ALD (triangles) 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 *right*) 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. 4*A*). In addition, the fact that siRNA for either GR or KLF15 shut down hormone-dependent induction of BCAT2 mRNA expression (Fig. 4*C*) 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. 4*D*). 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. 4*E*).

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. 5*A*), 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. 5*B*). However, this alteration in the concentrations of Glu to Gln was not affected by siRNA-mediated knockdown of KLF15 (Fig. 5*B*). In clear contrast, the concentrations of Val, Leu, and Ile were decreased after treatment with CVZ and affected by KLF15 knockdown (Fig. 5*B*). 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.

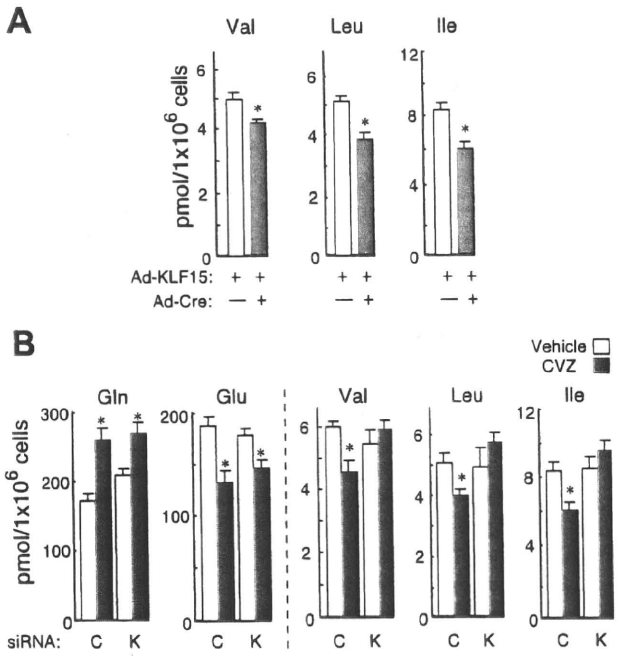

Fig. 5. Effects of glucocorticoid on intracellular concentration of amino acids. *A*: overexpression of KLF15 reduces the intracellular concentration of branched-chain amino acid (BCAA) in cardiomyocytes. Ad-Cre and Ad-KLF15 were infected in cultured rat cardiomyocytes, and the cells were cultured with fresh medium for 24 h. Measurement of amino acid concentration was performed as described in MATERIALS AND METHODS, and results are presented with means ± SE of 3 independent experiments. $^*P < 0.05$ vs. Ad-KLF15(−) cells. *B*: glucocorticoid differentially modulates amino acid concentration in rat cardiomyocytes. The cardiomyocytes were transfected with siRNA oligonucleotides for KLF15 (K) or control (C) as indicated and cultured for 24 h. Next, the cells were treated with vehicle or 100 nM CVZ for 24 h. Results are presented with means ± SE of 3 independent experiments. $^*P < 0.05$ vs. vehicle-treated cells.

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. 6*C*). 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. 6*D*). 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. 6*D* and data not shown). Glucocorticoid also induced mRNA expression of COX-1 and prostaglandin D_2 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 E_2 (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

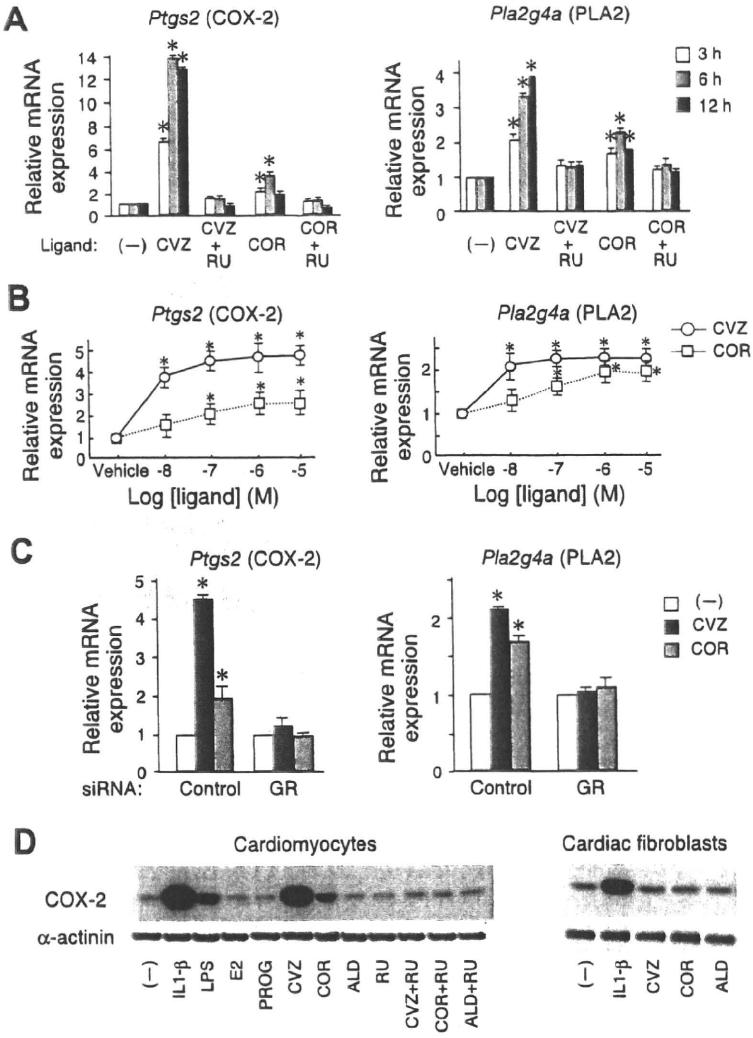

Fig. 6. Glucocorticoids regulate prostaglandin metabolism in the cardiomyocytes. *A*: time course of mRNA expression of *Ptgs2* (cyclooxigenase-2; COX-2) and *Pla2g4a* (cytoplasmic phospholipase A2; PLA2) in neonatal rat cardiomyocytes. The cells were cultured in the presence of each ligand for the indicated 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.

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 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 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 the gene expression program and control complexed metabolic milieus 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 coordinatedly regulate the gene expression program in concert with endocrine systems and contribute to maintenance of cardiac function.

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新しい心不全治療の標的分子としてのCdk9の役割とその活性制御機構

Activity Control Mechanism and Pathophysiological Significance of Cdk9 as a Novel Therapeutic Target of Heart Diseases

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Cdk-9はRNAポリメラーゼⅡのC末端ドメイン（CTD）のリン酸化を介してmRNA合成を包括的に活性化させ心筋細胞肥大を惹起しているだけでなく，転写レベルでのミトコンドリアエネルギー代謝制御因子であるPGC-1αの発現を抑制することによってエネルギー産生障害を引き起こし，心不全への進展にも深く関与している可能性が示唆される．このCdk9の活性は7SK snRNAを介するHEXIM1/2タンパク質との結合・解離によって制御されている．心臓に対する血行力学的なストレスによって活性化されているCdk9が心不全の発症を予防・遅延させうる新規治療戦略の標的分子となりうるか，筆者らの実験結果を踏まえて考察する．

key words　RNAポリメラーゼⅡ，転写伸長，Cdk9，心不全，HEXIM1/2，グルココルチコイド受容体

はじめに

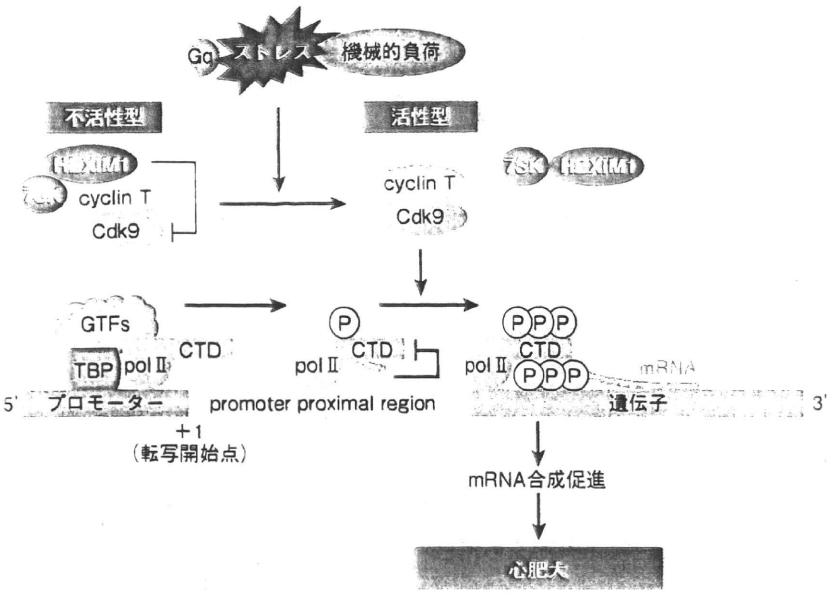
心臓を構成している心筋細胞は高度に特殊化した細胞であり，生後まもなく分裂能力を失う．また，心臓局所あるいは骨髄由来の幹細胞から新たに心筋細胞を再生する能力は限られている．したがって，持続性の高血圧や心筋梗塞による作業心筋の喪失に基づく血行力学的負荷に対して心臓は，新しい心筋細胞を作って心筋細胞の数を増やすのではなく，すでに存在する個々の心筋細胞容積の増大，すなわち“肥大”という形で反応する[1]．この心筋肥大反応は従来，物理的に壁厚の増大によって心筋壁応力を軽減するための適応反応と見なされてきた．しかし，肥大した心筋は，収縮力低下から内腔の拡大，壁のひ薄化を来し早晩心不全へと移行する．また，疫学的調査結果からも心肥大が心不全や心臓突然死の発症を増加させる独立した危険因子であるということが示されてきた．したがって，心肥大が必ずしも血行力学的な負荷に対する適応反応ではなく，心疾患の予後に悪影響を及ぼす不適切な反応で，それ自体が治療の対象になりうると考えられる．

心筋細胞の肥大，すなわち個々の心筋細胞容積の増大という現象は細胞あたりのRNA/タンパク質量の増加の結果，引き起こされると考える．しかし，これまで心肥大において観察される細胞あたりのRNA/タンパク質量の増加の分子メカニズムは十分に理解されていなかった．

Ⅰ CTDのリン酸化を介したRNAポリメラーゼⅡの転写機能制御

発生段階あるいは臓器特異的遺伝子発現は，主として“転写因子”によって制御されている．転写因子が標的遺伝子のプロモーター上に結合することによってクロマチン構造のリモデリングが起こり，メディエーター複合体の働きを介してRNAポリメラーゼⅡが基本転写因子とともにプロモーター上に取り込まれ，転写が開始されるという現象は広く知られている．しかし，これら一連の出来事は遺伝子の発現過程においてはプロローグにすぎない．その後の長い転写伸長反応（RNA鎖をつないでいく反応）と終結反応，さらにこれらと同時に進行するpre-mRNAのプロセシング（キャップ形成，スプライシング，ポリアデニル化）の段階を経て成熟したmRNAが合成されていく．近年，この転写の伸長段階における遺伝子発現制御に関してかなり研究が進んできた．そして，この転写の伸長段階における遺伝子発現制御の鍵を握っているのがRNAポリメラーゼⅡのC末端ドメイン（C-terminal domain：CTD）と呼ばれるドメインである．

RNAポリメラーゼⅡは12個のサブユニットからなる0.5MD以上の巨大な複合体であるが，その最大サブユニットRpb1のCTDには7つのアミノ酸（Tyr-Ser-Pro-Thr-Ser-Pro-Ser）の単位がヒトではじつに52回も繰り返された特殊な構造が存在している．このCTDの7つのアミノ酸の反復構造の中で特に2番目と5番目のセリンが転写伸長反応の進行過程で2つのcyclin依存性キナーゼによって順次リン酸化を受け，このリン酸化されたCTDを足場として様々な転写制御因子が取り込まれることによって，転写伸長反応や



■図1 Cdk9は心肥大反応に必須のRNAポリメラーゼⅡリン酸化酵素である
心肥大刺激は内因性Cdk9阻害物質である7SK snRNA(7SK small nuclear RNA)/HEXIM1を解離させることによってCdk9を活性化する．活性化されたCdk9はCTDをリン酸化することによってRNAポリメラーゼⅡ(polⅡ)を活性化する．

mRNAのプロセシングが制御されているというモデルが提唱されている[2]．

簡単に説明すると，まず非リン酸化型のRNAポリメラーゼⅡがプロモーター上に取り込まれて転写開始前複合体を形成し転写が開始する．転写開始直後にRNAポリメラーゼⅡのCTDの7つのアミノ酸の反復構造の中で5番目のセリンが基本転写因子TFⅡHのCdk7によってリン酸化される．mRNA鎖が50bpほど合成されたところで陰性の転写伸長因子の働きによってRNAポリメラーゼⅡはいったんmRNAの合成を休止する(promoter proximal pausing)．この間に5番目のセリンがリン酸化されたCTDを足場としてキャッピング酵素複合体が取り込まれ，作り始められたmRNAの5'側にキャッピングが行われる．その後，陽性転写伸長因子〔pTEFb(positive transcription elongation factor b)(Cdk-9/cyclin T1)〕のサブユニットであるCdk-9(cyclin-dependent kinase-9)によって2番目のセリンがリン酸化されると，陰性転写伸長因子が解離してRNAポリメラーゼⅡはmRNAの合成を再開する[3]．

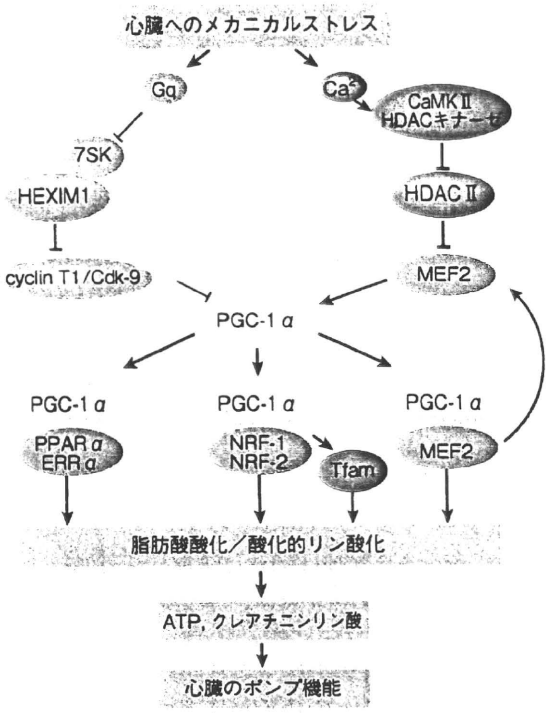
Ⅱ 心肥大におけるCdk9の役割

筆者らは心肥大において転写が非特異的に活性化されているという現象を見つけていたが[4]，大動脈縮窄による圧負荷に伴う心肥大やsmall Gタンパク質(Gq)やカルシニューリンの心筋特異的過剰発現による心肥大モデルマウスにおいて，高度リン酸化型のRNAポリメラーゼⅡの比率が増加していることが確認された．CTDのリン酸化は活発にmRNAを合成しているRNAポリメラーゼⅡの特徴であるから，この結果は心肥大においてRNAポリメラーゼⅡによるmRNA合成能が上昇していることを意味する．さらに，肥大した心筋において高度リン酸化型のRNAポリメラーゼⅡの比率が増加に伴って2つのRNAポリメラーゼⅡリン酸化酵素Cdk9とCdk7の活性の上昇も認められた．

そこで，培養心筋細胞を用いて，Cdk9とCdk7のRNAポリメラーゼⅡリン酸化と心肥大反応への関与を検討した．培養心筋細胞をET-1(endothelin-1)で刺激すると，RNAポリメラーゼⅡリン酸化と心筋細胞容積の増大(心肥大)が観察される．Cdk9阻害剤DRB(5,6-dichloro-1-beta-d-ribofuranosylbenzimidazole)やCdk9の優勢抑制型変異体によってCdk9の活性を抑制すると，ET-1によるRNAポリメラーゼⅡリン酸化や心肥大は抑制された．しかし，Cdk7の優勢抑制型変異体によってCdk7の活性を抑制してもRNAポリメラーゼⅡリン酸化や心肥大は抑制されなかったことから，Cdk7の活性は少なくとも*in vitro*での系では心筋細胞肥大には関与していないと考えられた．さらに，Cdk9のcyclinパートナーであるcyclin T1を心筋に過剰発現させたトランスジェニック(TG)マウスを作製したところcyclin T1の発現量に比例して心筋Cdk9活性の上昇と心筋肥大を認めたことから，Cdk9の活性化は心肥大の形成に必要かつ十分であることがわかった[5](図1)．

Ⅲ 心不全の病態形成におけるCdk9の役割

心肥大反応は，血行力学的負荷に対して心筋壁応力を軽減し，心拍出量を維持するための適応現象(代償性心肥大)とも見なせるが，負荷が取り除かれなければ不可避的に心不全に陥る．Cdk9は心筋に対する様々なストレス刺激に反応し活性化されるが，その活性は不全心においても高いま



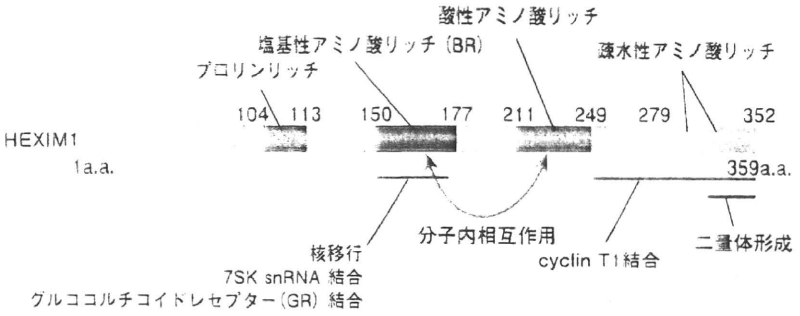
■図2 Cdk9は心臓におけるPGC-1 αの発現を抑制する
心臓におけるPGC-1 αの発現は，Ca^{2+}シグナル→CaMK II→HDAC II→MEF2経路によって正に制御されている．病的な心肥大過程においても，Ca^{2+}シグナル→CaMK II→HDAC II→MEF2経路は活性化されているにもかかわらず，PGC-1 αの発現は逆説的に減少している．病的な心肥大課程におけるCdk9の活性化は，PGC-1 αの発現を負に制御する．

ま維持され続けている．この現象は病的心肥大や心不全を呈する様々な遺伝子操作マウスモデルだけにとどまらずヒトにおいても確認された．すなわち，拡張型心筋症の患者から摘出された心筋組織において，Cdk9活性が健常心と比べて有意に上昇していた．このことは，Cdk9の活性化が単に心筋細胞肥大を惹起するだけでなく，代償性心肥大から不全心の移行にも深く関与している可能性を示唆している．

Cdk9の活性化がストレス刺激に対する適応反応なのか，あるいは有害な反応なのかを見極めるために負荷をかけた状態でのcyclin T1 TGマウスの心臓を観察した．cyclin T1 TGマウスは負荷がない定常状態では，心筋収縮機能が野生型と同程度に維持されていた．このcyclin T1 TGマウスを心筋特異的Gq TGマウスと掛け合わせることによりGq結合型受容体からのシグナルが恒常的に活性化されているcyclin T1 TGマウスは心筋壁の著明な菲薄化を呈し，生後4週齢までに全例心不全死した．また，大動脈縮窄による圧負荷をかけると術後早期の高い死亡率を呈し生存し得たものは，強い求心性心肥大（心室腔の容積はあまり増加せず心室壁が肥厚する）と心収縮力の低下を認めた．興味深いことに負荷をかけた状態でのcyclin T1 TGマウスの心筋では，心筋細胞のアポトーシスによる細胞死の増加を認めた．以上の結果から，ストレス刺激下での慢性的なCdk9の活性化が心筋細胞肥大のみならず，心筋収縮力の低下や心筋細胞のアポトーシスに対する感受性の亢進を引き起こし心不全へ進展に重要な役割を果たしていることが推測された[6]．

Cdk9を活性化によって引き起こされるストレス刺激下での心筋収縮不全やアポトーシスによる細胞死に対する感受性亢進の分子生物学的背景を探求するためにマイクロアレイを用いてcyclin T1 TGマウスの遺伝子発現変化を網羅的に解析した．Cdk9の活性化が純粋にすべての遺伝子の転写伸長反応を均等に活性化するだけならば，遺伝子発現様式には影響を及ぼさず包括的にmRNAの合成だけが増加しているという結果が期待された．しかし，実際には，cyclin T1の過剰発現により多彩な遺伝子発現様式の変化が観察された．驚いたことにミトコンドリアにおけるエネルギー代謝を司る遺伝子群，すなわち脂肪酸のβ酸化，TCAサイクル，呼吸鎖に関与する酵素群やミトコンドリアの抗酸化作用物質，ミトコンドリアDNAの複製・転写に関わる転写因子，ミトコンドリアのリボソームタンパク質などの発現が包括的に抑制されていた．遺伝子発現レベルだけでなく実際にcyclin T1 TGマウスの心臓ではミトコンドリアの呼吸鎖複合体Ⅰ～Ⅴの酵素活性がすべて有意に低下していた．この潜在的ミトコンドリア機能低下はcyclin T1 TGマウスの心臓がストレスに対して代償不全を引き起こしやすい現象を概念的に説明しうると考えられた．

ミトコンドリアの代謝機能に関与する遺伝情報はミトコンドリアと核のDNA双方にコードされている．ミトコンドリアDNAには13個の電子伝達系に関与する酵素の遺伝子が含まれているだけで，それ以外のエネルギー代謝を司る酵素の遺伝子は核のDNAに組み込まれている．この核内DNAからの遺伝子発現は心筋に豊富に存在する転写活性化因子PGC-1 α（PPAR γ coactivator 1 α）により包括的に制御されている．PGC-1 αの発現は，エネルギー需要の増加に応じて誘導され，心筋細胞では生後の成長過程や生理的運動負荷により発現量が増加することが知られている．PGC-1 α発現量の増加は心筋エネルギーリザーブを高め，疲れにくく突然の機械的仕事量の増加に耐え得る成熟した心筋細胞を構築する．反対に心肥大や不全心においては，PGC-1 αの発現量が低下していることから，これらの病態で観察される“エネルギー枯渇状態”の形成に深く関わっていることが示唆される．筆者らは，Cdk9の活性化による包括的なミトコンドリア代謝を司る遺伝子群の発現低下がPGC-1 αの機能異常もしくは発現低下によって説明されうると考えた．実際，PGC-1 αの発現はcyclin T1 TGマ



■図3 ヒトHEXIM1の一次構造
ヒトHEXIM1は359アミノ酸(a.a.)からなる核タンパク質．塩基性アミノ酸リッチな領域(BR)は核移行に必須であるほか，7SK snRNA，グルココルチコイドレセプター（GR），また，HEXIM1分子内の酸性アミノ酸リッチな領域と結合する．疎水性アミノ酸リッチな領域は，HEXIM1の二量体化および7SK snRNA存在下におけるcyclin T1との結合に重要．プロリンリッチな領域は，HEXIM1のCdk9抑制活性を負に調節する

ウスの心筋において約1/3まで減少していた．さらに，培養心筋細胞を用いて，Cdk9の活性化，PGC-1 αの発現量の低下，PGC-1 αの下流遺伝子発現の低下との因果関係を検討した結果，Cdk9の活性化によってPGC-1 αの発現量低下が起こり，続いて，PGC-1 αの下流にあるミトコンドリアエネルギー産生酵素の発現低下を招くこと，反対にPGC-1 αの発現を外因性に補うことによってCdk9の活性化に伴う心筋細胞のアポトーシスに対する感受性の亢進が救済された．以上の結果から，Cdk9の活性化はストレス下における心筋収縮力の低下，アポトーシスに対する感受性の亢進を引き起こしているということが明らかになった(図2)．

Ⅳ Cdk9の活性化の分子メカニズム

Cdk9は他のcyclin依存性キナーゼとは異なり，細胞周期の進行と直接関係しないユニークなメカニズムによって，そのキナーゼ活性が可逆的に調節されている．細胞核内のCdk9-cyclin T1ヘテロダイマーは7SK snRNA (7SK small nuclear RNA) および核タンパク質HEXIM1〔hexamethylene bisacetamide (HMBA) -inducible protein 1/CLP-1/MAQ1/EDG1〕と結合して7SK snRNPを形成することによって，そのキナーゼ活性を抑制される[7]．7SK snRNAはRNAポリメラーゼⅢによって転写される約330ヌクレオチドからなる豊富な(細胞あたり2×10^5コピー)核内低分子RNAで，脊椎動物で高度に保存されている．

HEXIM1は当初，ヒト血管平滑筋細胞において増殖抑制・分化誘導剤HMBAによってmRNAレベルで発現誘導される遺伝子としてクローニングされた．ヒトHEXIM1は359アミノ酸からなり，おおまかに4つのアミノ酸領域に分けられる(図3)．中央付近の塩基性アミノ酸リッチな領域(basic region；BR)は核移行シグナルとして機能するほか，7SK snRNAの非存在下でC末端寄りの酸性アミノ酸リッチな領域と分子内相互作用する．また，BRはRNA結合モチーフの1つとして知られるアルギニンリッチモチーフを形成し，7SK snRNAと結合する．この結合はBRと酸性アミノ酸リッチな領域との相互作用を阻害し，7SK snRNA依存的なHEXIM1の立体構造変化を引き起こし，cyclin T1との結合を誘導して7SK snRNPの形成に寄与する．C末端側の疎水性アミノ酸リッチな領域は，HEXIM1の二量体化および7SK snRNA存在下におけるcyclin T1との結合に重要である．N末端寄りのプロリンリッチな領域は，HEXIM1のCdk9抑制活性を負に調節する機能がある．HEXIM1のホモログHEXIM2はN末端側を除きHEXIM1と非常に相同性の高いアミノ酸配列を持っており，HEXIM1と同様なCdk9活性制御機構に関わる可能性が示唆されているが，多くの組織においてHEXIM1に比べて発現量が低く，その寄与は明らかでない．

7SK snRNPの解離・形成は可逆的で，不活性型Cdk9と活性型Cdk9の核内における量的なバランスの迅速な調節に重要な役割を果たしていると考えられている．最近，その制御機構として，hnRNP Qなどのpre-mRNAスプライシング因子が，7SK snRNAに結合することによってCdk9-cyclin T1とHEXIM1の結合を阻害し，転写伸長反応を促進するモデル[8]，また細胞内のATPがHEXIM1のBRに結合することによって7SK snRNPの解離を促進し，利用可能なエネルギーの量に応じてCdk9活性を調節するモデルが提唱されている．一方，筆者らはHEXIM1のBRが，核内レセプタースーパーファミリーに属する転写因子，グルココルチコイドレセプター(glucocorticoid receptor；GR)に直接結合し，グルココルチコイド応答遺伝子プロモーターへのGRのリクルートメントおよびGRと転写共役因子との相互作用を抑制して，ホルモンシグナル依存的なmRNA発現を抑制することを示した[9]．さらに，この特異的なmRNA発現抑制は，7SK snRNAに結合せずCdk9活性を抑制しないHEXIM1組換え変異体も引き起こすことから，Cdk9抑制とは独立した機構によることを示した．HEXIM1のGR結合，7SK snRNA結合はどちらもHEXIM1のBRに依存し，HEXIM1-GR複合体は核内で7SK snRNPと独立に存在することから，グルココルチコイドホルモン依存的なGRの核移行が，HEXIM1を介して7SK snRNPの解離を誘導しCdk9の活性バランスの制御に関わることも想定される．

HEXIM1の発現は幅広い組織・細胞において認められるが，その発現量は様々である．また，上述の血管平滑筋細胞以外でもHMBAやUV照射などのストレス刺激によって発現誘導が認められるほか，組織の発生段階においてもその発現量が制御されている．例えば，マウス心臓の発生段階において，出生前は高発現しているが，出生とともにその発現量は減少する．HEXIM1ノックアウトマウスは，胎生16.5日ころに心筋のZ帯が観察されなくなるなどの異常を来し，出生前に著名な左心室肥大を呈して致死となる[10]．このことは，出生前の心臓においてHEXIM1がCdk9活性を抑制することにより，無計画なRNA/タンパク質量の増加の結果引き起こされる心肥大，さらには心不全への進行をくい止めていると解釈することが可能である．HEXIM1/2はCdk9と安定な複合体を形成してその活性を抑制する，現在のところ唯一のCdk9特異的インヒビタータンパク質であり，その活性制御機構の詳細な解明は，Cdk9を中心とする転写伸長制御機構の理解の進展に寄与するであろうと注目されている．

おわりに

Cdk9は，後天性免疫不全症候群（エイズ）の原因ウイルスであるHIV-1の転写・複製に必要な宿主因子としても知られている．ウイルス由来の因子を直接の標的とした治療法は，高頻度の突然変異による薬剤耐性株の出現が大きな問題となるが，Cdk9などの宿主由来の因子を標的とした治療法，すなわちflavopiridol，seliciclib，DRBといったCdk9特異的阻害剤を応用した創薬により，この問題を軽減できると考えられてきた．しかしながら，Cdk9は，RNAポリメラーゼIIを介する遺伝子発現にとって普遍的に必要な酵素であり，この活性をおしなべて抑制することは細胞の正常な機能の破綻を引き起こすことが予想される．実際，上記のCdk9阻害剤は，ヒト末梢血由来T細胞などにおいてHIV-1の増殖を抑制するが，同時に無視できない細胞毒性を併せ持つ．Cdk9を心不全治療の標的分子として考えた場合においても，この問題は程度の差はあれ，未解決のまま同様に存在する．したがって，病的状態下における過剰な活性化を選択的に抑制するような治療が理想的である．その意味でも，Cdk9の活性そのものを抑制するよりはむしろ活性化を引き起こす上流のシグナル伝達経路を標的とした治療が好ましいと考えられる．心肥大あるいは心不全を引き起こすストレスシグナルがどのようにして7SK RNAを介したHEXIM1/2によるCdk9の抑制を解除し，過剰なCdk9の活性化を引き起こすのか，今後の解析が待たれる．

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for beginners

「転写がわかる わかる実験医学シリーズ」半田 宏：羊土社 (2002)

Bedside Teaching

アルデヒドと老化*

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アルデヒドと聞くとアルコール代謝を連想される方が多い．お酒を摂取すると，肝臓でエタノール→アセトアルデヒド→酢酸と代謝されていく(図1)．お酒を飲むと気分が良くなるのはエタノールの作用，反対に顔が紅潮し動悸がして気分が悪くなるのはアセトアルデヒドの作用である．お酒がよく飲めるヒトと飲めないヒトがいるが，これはアセトアルデヒドの代謝能力の違いによる．アセトアルデヒドの代謝は主としてALDH 2というアセトアルデヒド脱水素酵素が担っている．ALDH 2をコードしている遺伝子には遺伝子多型が存在する．活性型のALDH 2*1に対して点変異(E 487 K)が入っているALDH 2*2は不活性型で，その遺伝子産物は活性型のALDH 2*1に対して“優勢抑制型変異体”として働く．ALDH 2という酵素は4量体で働くが，4つのうちひとつでもALDH 2*2由来の蛋白が入ると酵素活性はなくなる．したがって，ALDH 2*1/ALDH 2*2のヘテロのヒトはALDH 2*1/ ALDH 2*1のホモのヒトに比べるとALDH 2の酵素活性としては1/2 ではなくて1/16まで低下しており，アセトアルデヒドの血液中濃度がすぐに高くなってしまうためにお酒が弱い(図2)．また，ALDH 2*2/ALDH 2*2のホモのヒトはまったくお酒が飲めない．成人してお酒を飲んでみたら一口だけで顔面が紅潮し心臓がどきどきして全く受け付けなかったというヒトは，ALDH 2*2/ALDH 2*2のホモである．日本人の4割のヒトがALDH 2*1/ALDH 2*2のヘテロで，1割のヒトがALDH 2*2/ALDH 2*2のホモである．つまり，10人に1人は，お酒が全く飲めないヒトということになる．ALDH 2の遺伝子多型は，遺伝子を調べなくても経験から自分で自覚できる．また，前腕にアルコールを浸したバンドエードを貼るという学生時代にやったあの簡単なパッチテストも実はALDH 2活性をみている検査である．

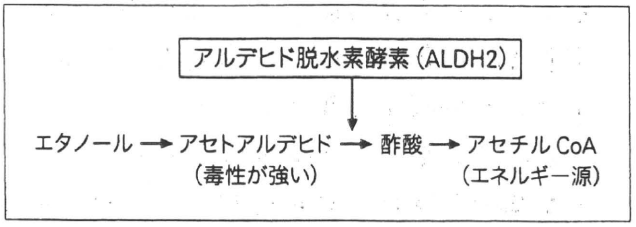

図1　アルコール代謝ALDH 2は飲酒時に生じるアセトアルデヒドの主要な代謝酵素である．

アルデヒドは，お酒を飲まなくても体の中で自然に生じている．食品中に含有される脂質の酸化劣化(酸化的分解過程)は，においや嘔吐，ふるえ，下痢などの中毒症状の原因ともなるが，これは活性酸素(ラジカル：注1)によって脂質の過酸化反応が進行した結果生じたアルデヒドなどの二次酸化生成物によるものである．

これと同様な反応が生体内でも生じる．細胞膜

注1：原子核を取り巻く電子は2つずつペアで同じ軌道に存在している(共有電子対)が，同じ軌道上にひとつしか電子を持たない不安定な状態の原子や分子のこと．安定上にある他の分子から電子を奪い安定化状態になろうとする．

* Aldehyde and Aging

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