Peroxisome proliferator-activated receptor-γ suppresses *CYP11B2* expression and aldosterone production

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Abstract

Peroxisome proliferator-activated receptor-γ (PPARγ) is a nuclear receptor for the antidiabetic agent thiazolidinedione, which exerts various physiological activities, independent of lowering blood glucose. However, the role of PPARγ in aldosterone production has not been clarified. The objective of this study was to investigate the effect of PPARγ on aldosterone synthase gene (*CYP11B2*) expression and aldosterone production. Localization of PPARγ expression in normal adrenal cortex was determined by immunohistochemistry. Aldosterone production and *CYP11B2* expression levels were determined using human adrenocortical carcinoma H295R cells. Pioglitazone suppressed angiotensin II-induced aldosterone secretion and *CYP11B2* expression. PPARγ was expressed in zona glomerulosa in human normal adrenal gland. PPARγ overexpression enhanced pioglitazone-mediated *CYP11B2* transrepression. The pioglitazone-mediated suppression of aldosterone secretion and *CYP11B2* expression were canceled by PPARγ L466A/E469A mutant. Pioglitazone also suppressed potassium-mediated *CYP11B2* induction, but not *N*6-2′-*O*-dibutyladenosine-3′,5′-cyclic monophosphate stimulation. Rosiglitazone and GW1929 also suppressed *CYP11B2* transactivation. Mutation analysis revealed that the Ad1/CRE element in *CYP11B2* 5′-flanking region was responsible for the pioglitazone-mediated transrepression. Pioglitazone suppressed ionomycin and a truncated constitutively active form Ca^{2+}/calmodulin-dependent kinase I (CaMKI)-mediated *CYP11B2* transcriptional activation. A CaMK inhibitor KN-93 attenuated pioglitazone-mediated *CYP11B2* transrepression. PPARγ suppresses *CYP11B2* expression and aldosterone secretion.

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Introduction

Nuclear receptor peroxisome proliferator-activated receptor-γ (PPARγ), which is targeted by the antidiabetic agent thiazolidinedione (TZD), is abundantly expressed in adipose tissue and acts as a key regulator for adipocyte differentiation. PPARγ also is expressed in various tissues involved in metabolic functions including skeletal muscle, liver, and pancreas (Heikkinen *et al.* 2007).

Additionally, roles of PPARγ in cardiovascular disorders including atherosclerosis/hypertension have been reported. PPARγ agonists prevent macrovascular events in type 2 diabetic patients, independent of glycemic control (Dormandy *et al.* 2005). PPARγ agonists also prevent hypertension in high-fat-fed Zuker rats (Walker *et al.* 1999) and renin/angiotensinogen double-transgenic mice (Ryan *et al.* 2004). Moreover, the human PPARγ dominant negative mutant induces hypertension and metabolic dysfunctions (Barroso *et al.* 1999), and vascular smooth muscle cell (VSMC)-specific PPARγ dominant negative mutant transgenic mice develop impaired vasodilation and hypertension (Sugawara *et al.* 2001). We have previously shown that PPARγ agonists suppress angiotensin II (AII) type 1 receptor (Halabi *et al.* 2008) and thromboxane receptor (Sugawara *et al.* 2002) in VSMCs as well as macrophage activation (Jiang *et al.* 1998, Ricote *et al.* 1998) and thromboxane synthase expression in macrophages (Ikeda *et al.* 2000). Thus, VSMCs and macrophages are important targets of PPARγ against atherosclerosis/hypertension. Additionally, some PPARγ agonist is reported to decrease AII-induced plasma aldosterone levels in Sprague–Dawley rats (Diep *et al.* 2002).

Aldosterone is an important factor in the progression of hypertension and vascular damage. Although adrenalectomy ameliorates vascular injury

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in AII/salt-treated rats, independent of systemic blood pressure, administration of aldosterone abolishes the adrenalectomy effects (Rocha *et al.* 2000, 2002). PPARγ is shown to affect several aldosterone regulatory factors including an increase in renin expression (Todorov *et al.* 2007) and body fluid volume by sodium absorption from the collecting ducts in the kidney (Guan *et al.* 2005), and a decrease in AII secretion from adipocytes (Harte *et al.* 2005). Despite the fact that some PPARγ agonists decrease plasma aldosterone levels in rats (Diep *et al.* 2002), direct effects of PPARγ on aldosterone secretion in the adrenal gland are unknown.

Steroid hormone biosynthesis is rapidly regulated by the translocation of substrate cholesterol from the outer mitochondrial membrane to the inside of mitochondria (Rainey *et al.* 2004). In contrast, chronic aldosterone secretion is limited by the expression of the aldosterone synthase gene, *CYP11B2*. In this study, we have investigated the roles of PPARγ on *CYP11B2* expression/aldosterone secretion using adrenocortical H295R cells.

Materials and methods

Reagents

Pioglitazone, rosiglitazone, GW1929, and GW9662 were purchased from Alexis Biochemicals (Farmingdale, NY, USA). Human AII and *N*6-2′-*O*-dibutyladenosine-3′, 5′-cyclic monophosphate (dbcAMP) were purchased from Sigma. KN-93 was purchased from Calbiochem (La Jolla, CA, USA). HX630 was kindly provided by Dr Kagechika (Tokyo Medical and Dental University).

Plasmids

The subcloned chimeric constructs containing the human *CYP11B2* genomic DNA and luciferase cDNA (pGL3-basic, Promega) were used for the transient transfection studies: −1521/+2-luc (harboring the *CYP11B2* 5′-flanking region from −1521 to +2 relative to the transcription start site upstream of the luciferase cDNA in pGL3-basic); −747/+2-luc; −135/+2-luc; −65/+2-luc. β-Galactosidase control plasmid in pCMV (pCMV-β-gal) was purchased from Clontech. Murine PPARγ1 expression vector (pCMX–PPARγ1) was kindly provided by Dr Umesono (Kyoto University). Truncated human Ca^{2+}/calmodulin-dependent kinase I (CaMKI, residues 1–295; Sun *et al.* 1996) and murine Nur-related factor 1 (NURR1) cDNA were cloned by PCR from normal human dermal fibroblast or murine pituitary AtT20 cell RNA and cloned into the pcDNA3 expression vector (Invitrogen; CaMKI-295–pcDNA3 and NURR1–pcDNA3). Several vectors were mutated using a QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA); activation function domain 2 (AF-2 domain) in pCMX–PPARγ1 from L466 to A and E469 to A (pCMX–PPARγ1 L466A/E469A); Ad5 element in −1521/+2-luc from 5′-CTCCAGCCTTGACCTT-3′ to 5′-CTCCAGCCTTGAtaTc-3′ (−1521/+2-luc-Ad5-mut); Ad1/CRE element in −1521/+2-luc from 5′-TGACGTGA-3′ to 5′-gGtaccGA-3′ (−1521/+2-luc-Ad1/CRE-mut; Bassett *et al.* 2004). Ad1/CRE element of *CYP11B2* gene promoter (CAGTTCTCCCATGACGTGATATGTTTCC) was inserted into the upstream of pGL3-promoter vector (Promega; Ad1/CRE-SV-Luc).

Cell culture

H295R cells were grown with 1:1 mixture of DMEM and Ham's F12 medium supplemented with 10% fetal bovine serum (FBS), insulin–transferrin–selenium-G supplements (Invitrogen), 1·25 mg/ml BSA (Sigma), 5·35 μg/ml linoleic acid (Sigma), 100 U/ml penicillin, and 100 μg/ml streptomycin. Cells were cultured in a humidified incubator at 37 °C with 5% CO_2. SW-13 cells were provided by Health Science Research Resources Bank, and were grown in Leibovitz's L-15 supplemented with 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin in a humidified incubator at 37 °C with air.

RNA preparation and quantitative reverse transcription-PCR

Human total RNAs from normal heart (pooled from three male Caucasians, aged 30–39; cause of death: trauma), liver (from a 51-year-old male Caucasian; cause of death: sudden death), kidney (from a 40-year-old female Caucasian; cause of death: sudden death), skeletal muscle (pooled from two male/female Caucasians, aged 43 and 46; cause of death: sudden death), and adrenal gland (pooled from 62 male/female Caucasians, aged 15–61; cause of death: sudden death) were obtained from Clontech. When H295R cells were grown to 80% confluence in six-multiwell plates, they were exposed to pioglitazone for 48–72 h in DMEM supplemented with 1% stripped FBS. Then the cells were treated with AII, KCl, or dbcAMP. H295R and SW-13 cell total RNA were extracted using TaKaRa FastPure RNA kit (Takara Bio, Ohtsu, Japan) according to the manufacturer's instructions. Total RNAs were subjected to reverse transcription reaction using PrimeScript Reverse Transcriptase (Takara Bio) with random 6 mer and oligo dT primers according to the manufacturer's instructions. Thereafter, obtained templates were used for quantitative real-time PCR (95 °C, 3 min for 1 cycle; 95 °C, 15 s; 60 °C, 10 s; 72 °C,

20 s for 40 cycles) with iQ Supermix (for *CYP11B2*) or iQ SYBR Green Supermix (for others; Bio-Rad) by DNA Engine thermal cycler attached to Chromo4 detector (Bio-Rad). The following primer and TaqMan probe sequences were used: PPARγ (forward, 5′-GACCTGAAACTTCAAGAGTACC-3′, reverse, 5′-TGAGGCTTATTGTAGAGCTGAG-3′), *CYP11B2* (forward, 5′-GGCAGAGGCAGAGATGCTG-3′, reverse, 5′-CTTGAGTTAGTGTCTCCACCAGGA-3′, probe, 5′-CTGCACCACGTGCTGAAGCACT-3′), β-actin (forward, 5′-CCAACCGCGAGAAGATGACC-3′, reverse, 5′-CCAGAGGCGTACAGGGATAG-3′), calmodulin (CaM) 1 (forward, 5′-AACAGAAGCTGAATTGCAGG-3′, reverse, 5′-AATTCGGGGAAGTCAATGG-3′), CaM2 (forward, 5′-GATGAAATGATCAGGGAAGC-3′, reverse, 5′-CAAGGTCTTCACTTTGCTGTC-3′), CaM3 (forward, 5′-GATGGCCAGAAAGATGAAGG-3′, reverse, 5′-TGATGTAGCCATTCCCATCC-3′), Ca^{2+}/CaMK I (sense, 5′-AAGGCAGCATGGAGAATGAG-3′, reverse, 5′-CTACAATGTTGGGGTGCTTG-3′), and CaMKIV (forward, 5′-TGCTGCAGATGCCGTTAAAC-3′, reverse, 5′-AGATCACGATGGACAATCCC-3′).

Immunohistochemistry

Immunohistochemistry of normal human adrenal cortex from autopsy files of Tohoku University Hospital was performed by the streptavidin–biotin amplification method using Histofine kit (Nichirei, Tokyo, Japan). Antigen retrieval was performed by heating the slides in an autoclave at 120 °C for 5 min in citric acid buffer (2 mM citric acid and 9 mM trisodium citrate dehydrate (pH 6·0)). Rabbit polyclonal antibody raised against PPARγ was as previously described (Sato *et al.* 2004). The primary antibody was diluted at 1:3000, and the antigen–antibody complex was visualized with 3,3′-diaminobenzidine (DAB) solution (1 mM DAB, 50 mM Tris–HCl buffer (pH 7·6), and 0·006% H_2O_2). As a negative control, normal mouse or rabbit IgG was used instead of the primary antibody. Some specimens were stained with hematoxylin. Peptide pre-absorption of the antibody was performed as described previously (Sato *et al.* 2004).

Protein preparation and western immunoblot analysis

Nuclear protein was prepared from H295R cells using Nuclear Extract kit (Active Motif, Carlsbad, CA, USA) according to the manufacturer's instructions. The denatured samples were separated on an SDS–polyacrylamide gel and transferred onto polyvinylidene fluoride (PVDF) membrane (Bio-Rad; Uruno *et al.* 2004, 2005, 2008). The membranes were then blocked with 5% nonfat dry milk and probed with primary antibody for PPARγ (Sato *et al.* 2004; diluted at 1:1000), and HRP-conjugated antibody (GE Healthcare, Waukesha, WI, USA). The bands were visualized with ECL-plus reagent (GE Healthcare). Pre-absorption test was performed for PPARγ using the blocking peptide.

Measurement of aldosterone concentration

When H295R cells were grown to confluence in 24-multiwell plates, they were exposed to pioglitazone for 48–72 h in DMEM supplemented with 1% stripped FBS. AII was then added to the media at a concentration of 100 nmol/l, and the cells were incubated for 2 h. Next, the media were freshly changed, and the cells were further incubated in the presence of pioglitazone and AII for 2 h. Aldosterone concentrations of the media were thereafter measured by Aldosterone EIA kit (Cayman Chemical, Ann Arbor, MI, USA) according to the manufacturer's instructions.

Transient transfection and luciferase assay

When H295R cells were grown to 80% confluence in 24-multiwell plates, they were transiently transfected with 200 ng luciferase-reporter plasmids and 100 ng pCMV-β-gal using Lipofectamine LTX and Plus reagent (Invitrogen) for 48 h according to the manufacturer's instructions. In some experiments, expression vectors including pCMX–PPARγ1 (100 ng, if not otherwise specified), pCMX–PPARγ1 L466A/E469A (100 ng), or pcDNA3–CaMKI-295 (200 ng) also were transfected. The media were changed to DMEM supplemented with 1% charcoal/resin-treated (stripped) FBS, and the cells were incubated with or without PPARγ agonists for the indicated times. Then the cells were treated with AII (100 or 200 nmol/l), KCl (10·4 mmol/l, 16 mEq/l of K^+), dbcAMP (1 mmol/l), or ionomycin (1 μmol/l). After appropriated treatments, they were washed with PBS, and the cell extracts were prepared using Glo Lysis buffer (Promega). Luciferase activity was measured using Bright-Glo reagents (Promega), and β-galactosidase activity was simultaneously measured. Data were normalized by β-galactosidase activity.

Measurement of intracellular calcium

H295R cells (4×10^4) were seeded into 96-well plates, and the cells were incubated for 24 h. They were transiently transfected with 20 ng pCMX–PPARγ1 using Lipofectamine LTX and Plus reagent (Invitrogen) for 48 h, then were exposed to 3 μmol/l pioglitazone in DMEM supplemented with 1% strriped FBS for 24 h. Thereafter, the cells were loaded with Fluo4-AM (Dojindo, Kumamoto, Japan; 5 μg/ml) in the presence of 1·25 mmol/l probenecid (Dojindo) and 0·04% Pluronic F-12 (Dojindo) for 1 h.

They were then washed with PBS, and the recording medium containing 1·25 mmol/l probenecid, and AII (100 nmol/l) or KCl (40 mmol/l) was added to the media. The change of intracellular calcium was determined by fluorescent intensity (excitation at 485 nm, emission at 535 nm).

Statistical analysis

All data are presented as mean ± S.E.M. Statistical analyses were performed with ANOVA followed by Fisher's least significant difference *post hoc* test.

Results

PPARγ expression in normal adrenal gland and H295R cells

We first examined PPARγ expression levels in the normal human adrenal gland and H295R cells. *PPARγ* mRNA expression in the adrenal gland was lower than that in the kidney and heart, while higher than that in the liver, skeletal muscle, and H295R cells, and was rarely expressed in SW-13 adrenocortical carcinoma cells (Fig. 1A). Next, we determined the localization of PPARγ in the normal human adrenal cortex by immunohistochemistry. As shown in Fig. 1B (left panel), PPARγ was predominantly expressed in zona glomerulosa, while little in zona fasciculata and reticularis. The nuclear staining of human adrenal cortex with the PPARγ antibody was diminished with pre-absorption by its antigen peptide (Fig. 1B, right panel). Western blot analysis using anti-PPARγ antibody also revealed the expression of PPARγ protein in H295R cells nuclear protein, the band of which was abrogated by pre-absorption with the blocking peptide (Fig. 1C).

Effects of pioglitazone on aldosterone secretion/*CYP11B2* expression

We next examined the effect of a TZD pioglitazone on aldosterone secretion/*CYP11B2* expression. AII treatment increased aldosterone secretion from H295R cells into the media, and pioglitazone co-treatment suppressed the AII-induced aldosterone secretion (Fig. 1D). To evaluate the effect of *CYP11B2* gene-mediated regulation of aldosterone secretion, but not steroidogenic acute regulatory protein-mediated acute phase secretion, the media were changed once 2 h after AII stimulation. AII treatment also induced *CYP11B2* mRNA as well as gene transcriptional activity in H295R cells, and pioglitazone co-treatment reduced the AII-mediated inductions (Fig. 1E and F).

Effects of PPARγ on aldosterone secretion/*CYP11B2* expression

We next examined the effect of PPARγ overexpression on *CYP11B2* transcriptional activity using previously described promoter/reporter constructs (Bassett *et al.* 2004). As shown in Fig. 2A, PPARγ overexpression attenuated the AII-mediated *CYP11B2* transactivation both in the absence or presence of pioglitazone in a dose-dependent manner. We next examined the effects of PPARγ agonists/antagonist on *CYP11B2* transcriptional activity. As shown in Fig. 2B, the AII-mediated *CYP11B* transactivation was also suppressed by rosiglitazone and GW1929 to a similar extent as pioglitazone. Moreover, the pioglitazone-mediated *CYP11B* transrepression was abrogated by a PPARγ antagonist, GW9662 (Fig. 2C).

We next investigated the effect of PPARγ AF-2 domain mutation on *CYP11B2* transcriptional activity/aldosterone secretion. In the presence of wild-type PPARγ, pioglitazone suppressed both the AII-mediated *CYP11B2* transactivation (Fig. 2D) and aldosterone secretion (Fig. 2E), while the pioglitazone-mediated suppression was canceled in the presence of PPARγ L466A/E469A mutant. Next, we examined the role of retinoid X receptor (RXR) on *CYP11B2* transcriptional activity. As shown in Fig. 2F, an RXR agonist HX630 also suppressed the AII-induced *CYP11B2* transcriptional activity and additively decreased the pioglitazone-mediated transrepression. These data suggest that pioglitazone suppressed *CYP11B2* transcriptional activity and aldosterone secretion through PPARγ.

Pharmacological properties of pioglitazone on *CYP11B2* transcriptional activity

We next determined the pharmacological properties of pioglitazone on the AII-induced *CYP11B2* transcriptional activity. Time-course analysis revealed that pioglitazone suppressed the AII-mediated *CYP11B2* transactivation from 0·5 h, with maximal suppression at 24 h (Fig. 3A). Pioglitazone suppressed the AII-mediated *CYP11B2* transactivation in a concentration-dependent manner (Fig. 3B).

We next examined whether pioglitazone suppresses potassium- and cAMP-mediated *CYP11B2* expression. In addition to AII, potassium and the cAMP analogue dbcAMP increased both *CYP11B2* transcriptional activity (Fig. 3C) and mRNA expression (Fig. 3D). Pioglitazone suppressed not only the AII- but also the potassium-mediated upregulation of *CYP11B2* transcriptional activity (Fig. 3C) and mRNA expression (Fig. 3D), while it did not suppress the dbcAMP-mediated stimulation.

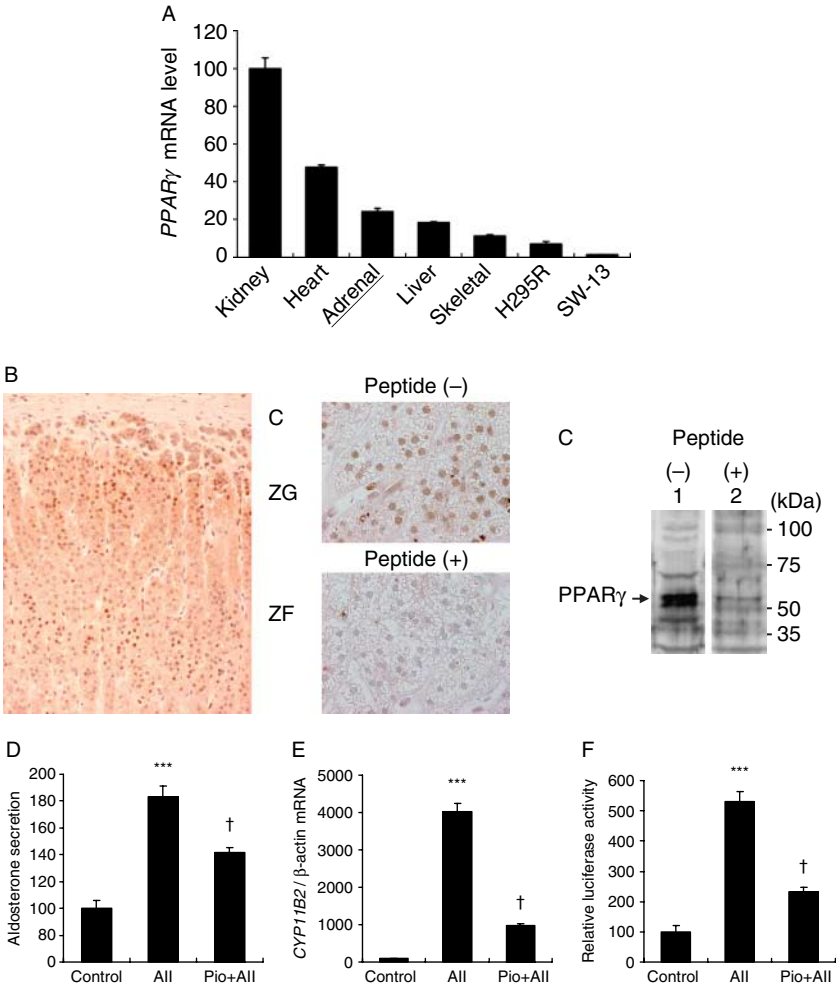

Figure 1 PPARγ expression and effect of pioglitazone on aldosterone production. (A) Expression of *PPARγ* mRNA in human organs, H295R, and SW-13 cells. Data represent mean±S.E.M. ($n=3$), percent of kidney group, copies of PPARγ cDNA as determined by quantitative RT-PCR per 1 ng total RNA. (B) Localization of PPARγ in human adrenal cortex determined by immunohistochemistry using anti-PPARγ antibody (left panel). Human adrenal cortex was stained by hematoxylin and anti-PPARγ antibody with (lower) or without (upper) pre-absorption by its antigen peptide (right panel). (C) PPARγ protein expression in H295R cell nuclear protein determined by western immunoblot analyses. Immunoblot analyses were performed using anti-PPARγ antiserum in the absence (lane 1) or presence (lane 2) of blocking peptide. (D) Effect of pioglitazone on aldosterone secretion into the media from H295R cells. H295R cells were treated with pioglitazone (Pio; 10 μmol/l, 72 h) and AII (100 nmol/l, 4 h). Data represent mean±S.E.M. ($n=6$), percent of control. (E) Effect of pioglitazone on *CYP11B2* mRNA expression in H295R cells. Cells were treated with pioglitazone (Pio; 10 μmol/l, 72 h) and AII (100 nmol/l, 6 h). Data represent mean±S.E.M. ($n=4$), percent of control, normalized by β-actin mRNA levels. (F) Effect of pioglitazone on *CYP11B2* transcriptional activity. H295R cells transfected with −1521/+2-luc and pCMV-β-gal were treated with pioglitazone (Pio; 10 μmol/l, 48 h) and AII (100 nmol/l, 4 h). Data represent mean±S.E.M. ($n=4$), percent of control. ***$P<0{\cdot}001$ versus control. †$P<0{\cdot}001$ versus AII.

Role of Ad1/CRE element on pioglitazone-mediated *CYP11B2* transrepression

To explore the mechanism(s) for the pioglitazone-mediated *CYP11B2* transrepression, we next examined the transcriptional activity of the *CYP11B2* 5′-flanking region using its deletion mutants. Both the AII-induced (Fig. 4A) and potassium-induced (Fig. 4B) *CYP11B2* transactivation and the pioglitazone-mediated suppression were observed in −1521/+2-luc, −747/+2-luc, and −135/+2-luc. However, the AII- and potassium-mediated *CYP11B2* transactivation and the

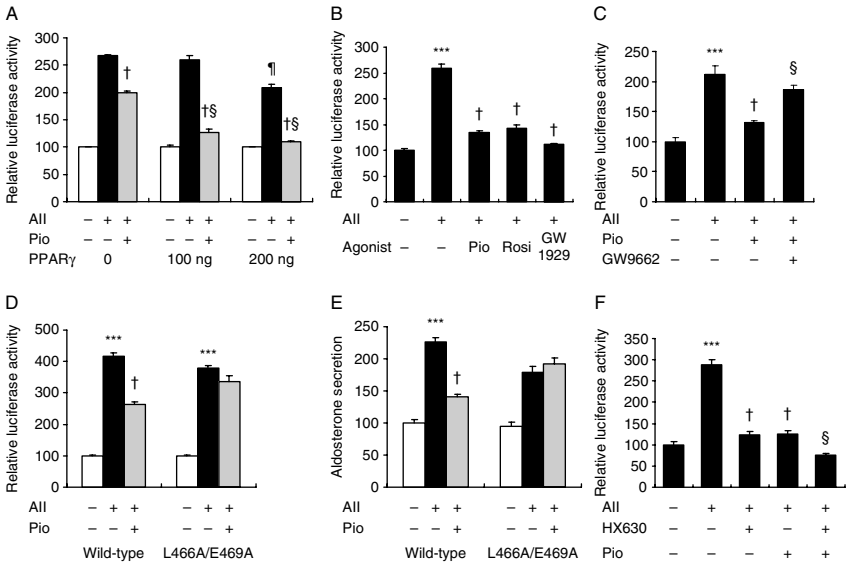

Figure 2 Role of PPARγ on *CYP11B2* regulation. Effects of PPARγ overexpression (A), PPARγ agonists (B), PPARγ antagonist (C), and PPARγ1 L466A/E469A mutant (D) on *CYP11B2* transcriptional activity. H295R cells transiently transfected with −1521/+2-luc, pCMV-β-gal, and PPARγ expression vectors (A, 0–200 ng, at indicated dose; B–D, 100 ng) were treated with reagents (A, 10 μmol/l pioglitazone (Pio), 72 h; B–D, 3 μmol/l each Pio, rosiglitazone (Rosi), or GW1929, 20 μmol/l GW9662, 48 h) and AII (100 nmol/l, 4 h). Data represent mean±S.E.M. ($n=4$), percent of control. (E) Effect of PPARγ1 L466A/E469A mutant on aldosterone secretion. H295R cells transiently transfected with −1521/+2-luc, pCMV-β-gal, and PPARγ expression vectors (100 ng) were treated with Pio (3 μmol/l, 48 h) and AII (100 nmol/l, 4 h). Data represent mean±S.E.M. ($n=4$), percent control. (F) Effect of RXR on *CYP11B2* transcriptional activity. H295R cells transiently transfected with −1521/+2-luc, pCMV-β-gal, and pCMX–PPARγ1 were treated with Pio (10 μmol/l, 24 h), HX630 (10 μmol/l, 24 h), and AII (100 nmol/l, 4 h). Data represent mean±S.E.M. ($n=4$), percent of control. *Wild-type*, pCMX–PPARγ1; *L466A/E469A*, pCMX–PPARγ1 L466A/E469A. (A) $^{\dagger}P<0{\cdot}001$ versus AII. $^{\P}P<0{\cdot}001$ versus AII without PPARγ expression vector. $^{\S}P<0{\cdot}001$ versus AII plus Pio without PPARγ expression vector. (B and D–F) $^{***}P<0{\cdot}001$ versus control. $^{\dagger}P<0{\cdot}001$ versus AII. (C) $^{***}P<0{\cdot}001$ versus control. $^{\dagger}P<0{\cdot}001$ versus AII. $^{\S}P<0{\cdot}001$ versus Pio plus AII.

pioglitazone-mediated transrepression were not observed in −65/+2-luc, which lacks both the Ad5 and Ad1/CRE elements. We thereafter examined the transcriptional activity of the Ad5 and Ad1/CRE elements in *CYP11B2* 5′-flanking region using their mutants. As shown in Fig. 4C, mutation of the Ad5 element (−1521/+2-luc-Ad5-mut) partially abrogated both the AII- and potassium-induced *CYP11B2* transcriptional activity comparing to the wild-type −1521/+2-luc, but did not cancel the pioglitazone-mediated *CYP11B2* transrepression (AII, 40% suppression in wild-type versus 34% suppression in Ad5-mut; potassium, 42% suppression in wild-type versus 46% suppression in Ad5-mut). On the other hand, mutation of the Ad1/CRE element (−1521/+2-luc-Ad1/CRE-mut) strongly reduced both the AII- and potassium-induced *CYP11B2* transcriptional activity, and abrogated the pioglitazone-mediated *CYP11B2* transrepression (AII, 40% suppression in wild-type versus 13% suppression in Ad1/CRE-mut; potassium, 42% suppression in wild-type versus 19% suppression in Ad1/CRE-mut). AII also increased the transcriptional activity of Ad1/CRE-SV-Luc, and pioglitazone suppressed the AII-mediated induction (Fig. 4D). AII or pioglitazone did not alter the transcriptional activity of pGL3-promoter (Fig. 4D). These data suggest that the Ad1/CRE element is probably responsible for the pioglitazone-mediated *CYP11B2* transrepression. We next examined the effect of pioglitazone on the AII-induced expression of nerve growth factor-induced clone B (NGFIB) and NURR1, both of which are nuclear orphan receptors binding to the Ad5 element and positively regulate *CYP11B2* transcriptional activity. Although AII increased both *NGFIB* and *NURR1* mRNA expression, pioglitazone did not affect their expression (Supplementary Figure 1, see section on supplementary data given at the end of this article), indicating their lesser involvement in the transrepression. Consistent with the notion, pioglitazone had little effect on the NURR1-mediated *CYP11B2* transactivation (Fig. 4E).

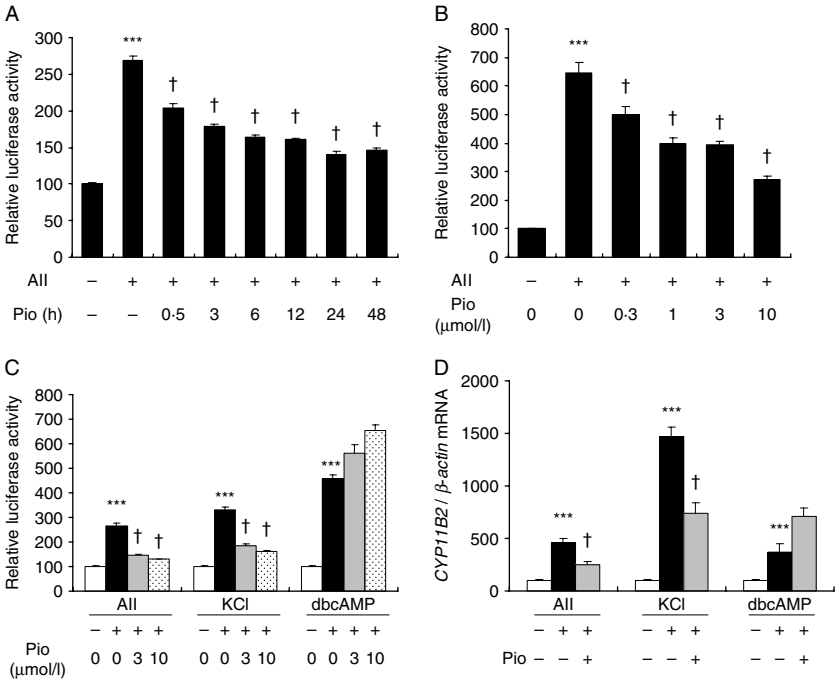

Figure 3 Pharmacological analyses of pioglitazone on H295R cells. (A and B) Time-course (A) and dose–response (B) effects of pioglitazone (Pio) on AII-induced *CYP11B2* transcriptional activity. (C) Effect of pioglitazone (Pio) on potassium- and dbcAMP-mediated *CYP11B2* transactivation. H295R cells transiently transfected with −1521/+2-luc, pCMV-β-gal, and pCMX–PPARγ1 were treated with Pio (A, 3 μmol/l, the indicated duration before AII addition; B and C, the indicated concentration, 24 h) and AII (A and C, 100 nmol/l; B, 200 nmol/l, 4 h), and KCl (10·4 mmol/l, 16 mEq/l of K^+, 8 h) or dbcAMP (1 mmol/l, 4 h) was thereafter added. Data represent mean±S.E.M. (n=4), percent of control. (D) Effect of Pio on potassium- and dbcAMP-induced *CYP11B2* mRNA expression. H295R cells transfected with pCMX–PPARγ1 were treated with Pio (3 μmol/l, 48 h), AII (100 nmol/l, 6 h), KCl (10·4 mmol/l, 16 mEq/l of K^+, 12 h), or dbcAMP (1 mmol/l, 6 h). mRNA expression was determined by quantitative RT-PCR. Data represent mean±S.E.M. (n=4), percent of control, normalized by β-actin mRNA levels. ***$P<0·001$ versus control. †$P<0·001$ versus AII, KCl, or dbcAMP.

Role of pioglitazone on calcium signaling

Since the Ad1/CRE element is essential for the AII- and potassium-mediated *CYP11B2* transactivation via the Ca^{2+}–CaM–CaMKI signaling pathway (Clyne *et al.* 1997, Pezzi *et al.* 1997, Condon *et al.* 2002), we therefore investigated the role of pioglitazone on this pathway. As shown in Fig. 5A and B, both AII and potassium increased intracellular calcium ion concentration in H295R cells. Although pioglitazone did not affect the AII-mediated intracellular Ca^{2+} increase, it enhanced the potassium-mediated one. We next examined the effect of pioglitazone on the Ca^{2+}-mediated *CYP11B2* transcriptional activity. As shown in Fig. 5C, ionomycin increased *CYP11B2* transcriptional activity, while pioglitazone diminished the increase. We next examined the effects of pioglitazone on *CaMs* mRNA expression. As shown in Fig. 5D, the mRNA level of CaM3 was significantly higher than that of CaM1 and CaM2. Pioglitazone weakly increased the mRNA expression of both CaM1 and CaM2, while it did not affect that of CaM3. These data indicate that pioglitazone partially enhances, but not suppresses, the Ca^{2+}–CaM signals.

Role of CaMK on pioglitazone-mediated *CYP11B2* transrepression

Since CaMKI is known to stimulate *CYP11B2* transcriptional activity through the Ad1/CRE element downstream of the Ca^{2+}–CaM signals (Condon *et al.* 2002), we next examined the role of CaMKI on the pioglitazone-mediated *CYP11B2* transrepression. In addition to CaMKI, CaMKIV is also reported to increase *CYP11B2* transcriptional activity weakly (Condon *et al.* 2002). Therefore, we evaluated the expression level of CaMKI and IV. As shown in Fig. 6A, the mRNA expression of CaMKI in H295R cells was much higher than that of CaMKIV. Pioglitazone did not affect their mRNA expression level. Overexpression of a truncated

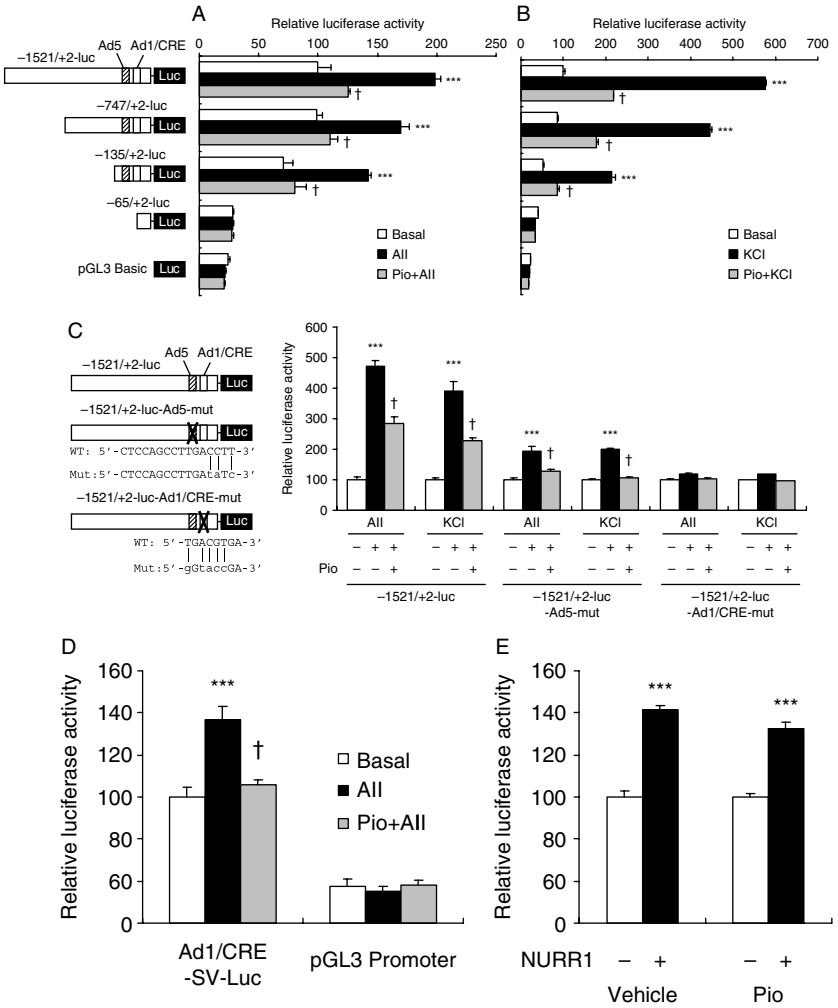

Figure 4 Effect of *CYP11B2* 5′-flanking region mutant on pioglitazone-mediated *CYP11B2* transrepression. (A–D) H295R cells transfected with luciferase-reporter vectors, pCMV-β-gal, and pCMX–PPARγ1 were treated with pioglitazone (Pio; 3 μmol/l, 48 h), AII (100 nmol/l, 4 h), or KCl (10·4 mmol/l, 16 mEq/l of K^+, 8 h). Data represent mean ± S.E.M. ($n=4$), percent of basal in −1521/+2-luc group (A and B), basal in each luciferase-reporter groups (C), or basal in Ad1/CRE-SV-Luc group (D). ***$P<0·001$ versus basal. †$P<0·001$ versus AII or KCl. (E) Effect of Pio on NURR1-mediated *CYP11B2* transcriptional activity. H295R cells were transfected with −1521/+2-luc, pCMV-β-gal, pcDNA3 (*NURR1* (−)), or NURR1–pcDNA3 (*NURR1* (+)). Cells were treated with Pio (3 μmol/l, 48 h). Data represent mean ± S.E.M. ($n=4$), percent of NURR1 (−). *** $P<0·001$ versus NURR1 (−).

constitutively active form of CaMKI (CaMKI-295) increased *CYP11B2* transcriptional activity (Fig. 6B), and pioglitazone completely abrogated the increase. As shown in Fig. 6C, the AII- or potassium-mediated *CYP11B2* transactivation was not observed in the presence of a CaMK inhibitor KN-93. Additionally, the pioglitazone-mediated transrepression was also canceled in the presence of KN-93. These data indicate that pioglitazone suppresses *CYP11B2* transactivation probably via the suppression of signal transduction from CaMKI to *CYP11B2* promoter.

Discussion

The present data demonstrate that PPARγ decreases *CYP11B2* expression/aldosterone secretion through the suppression of CaMKI function that stimulates the Ad1/CRE element in *CYP11B2* 5′-flanking region in the human adrenal H295R cells.

Expression of PPARγ in mouse (Kliewer *et al.* 1994) and human (Ferruzzi *et al.* 2005) adrenal gland has previously been demonstrated. Although PPARγ agonists are reported to suppress androgen

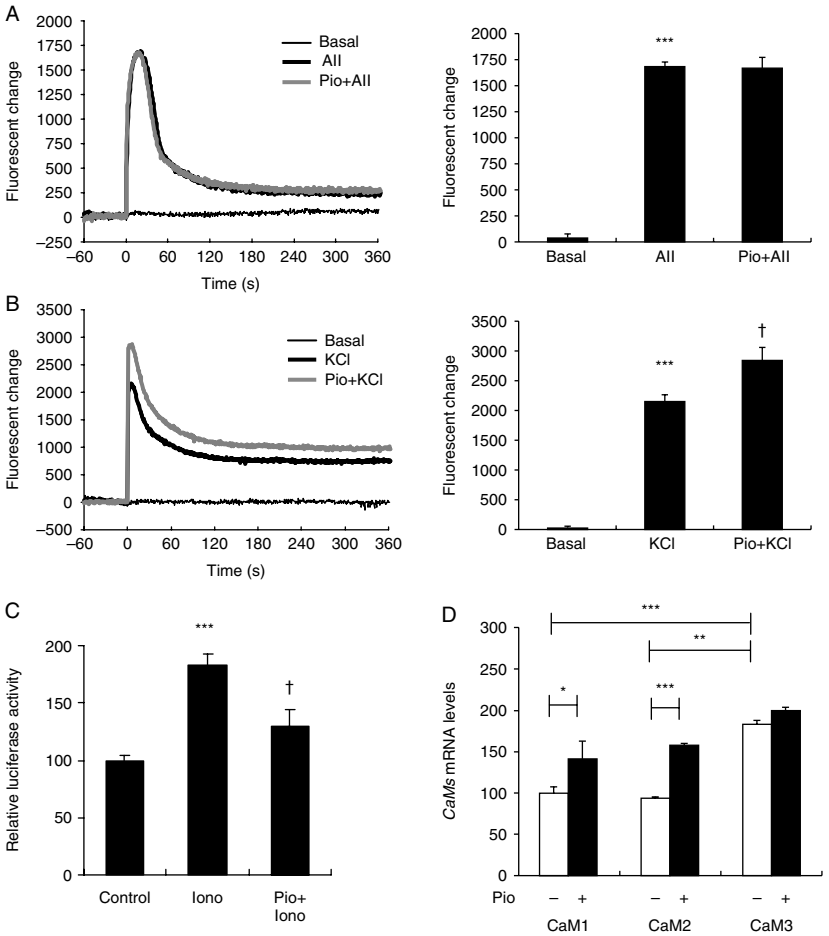

Figure 5 Role of pioglitazone on calcium regulation in H295R cells. (A and B) Effect of pioglitazone on intracellular calcium ion concentration in H295R cells determined by Fluo4-AM. H295R cells transfected with pCMX–PPARγ1 were treated with or without pioglitazone (Pio; 3 μmol/l, 24 h). After loading with Fluo4-AM, cells were treated with AII (100 nmol/l) or KCl (40 mmol/l), and the fluorescence change was monitored. Data represent mean (the left panels, $n=5$–6) or mean±S.E.M. (the right panels, $n=5$–6), the fluorescence change from time 0, arbitorary units. $^{***}P<0{\cdot}001$ versus basal. $^{\dagger}P<0{\cdot}001$ versus KCl. (C) Effect of Pio on ionomycin-induced *CYP11B2* transcriptional activity. H295R cells were transfected with −1521/+2-luc, pCMV-β-gal, and pCMX–PPARγ1. Cells were treated with Pio (3 μmol/l, 48 h) and ionomycin (1 μmol/l, 4 h). Data represent mean±S.E.M. ($n=4$), percent of control. $^{***}P<0{\cdot}001$ versus control. $^{\dagger}P<0{\cdot}001$ versus ionomycin. (D) Effect of Pio on *CaMs* mRNA expression. H295R cells transfected with 200 ng pCMX–PPARγ1 were treated with (filled columns) or without (open columns) Pio (3 μmol/l, 48 h). Data represent mean±S.E.M. ($n=4$), percent of CaM1 without Pio group, normalized by β-actin mRNA levels. $^{***}P<0{\cdot}001$, $^{**}P<0{\cdot}01$, $^{*}P<0{\cdot}05$. Iono, ionomycin.

production and expression of 17α-hydroxylase (CYP17) and 3β-hydroxysteroid dehydrogenase type 2 (HSD3B2) in H295R cells (Kempna *et al.* 2007), as well as inhibit adrenal cancer cell line proliferation (Betz *et al.* 2005, Ferruzzi *et al.* 2005), these effects are shown to be independent of PPARγ. Thus, the function of PPARγ in adrenal gland still remains uncertain. The PPARγ expression level in the normal human adrenal gland is reported to be lower than that in carcinoma as determined by immunohistochemistry (Ferruzzi *et al.* 2005). In this study, we also demonstrated that the expression level of PPARγ in the human adrenal gland was higher than that in the liver and skeletal muscle (Fig. 1A). Additionally, immunohistochemistry of the normal human adrenal cortex revealed that PPARγ was predominantly localized in the zona glomerulosa (Fig. 1B). We therefore explored the roles of PPARγ in aldosterone production and *CYP11B2* expression in the human adrenal cells.

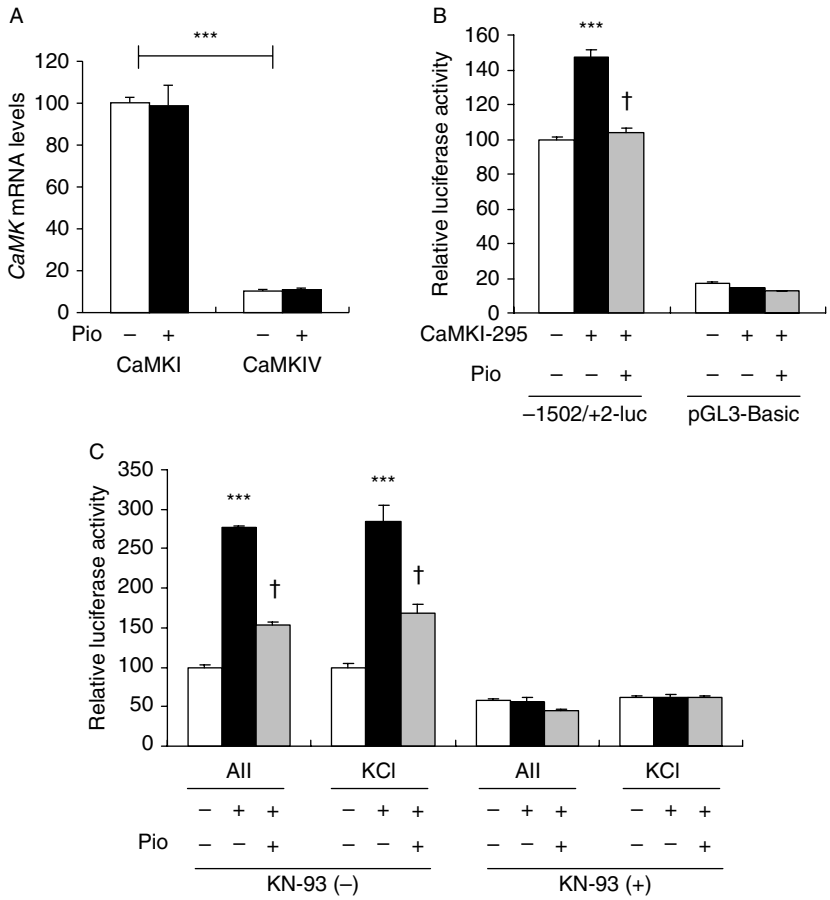

Figure 6 Effect of CaMK on pioglitazone-mediated *CYP11B2* transrepression. (A) CaMKs mRNA expression. H295R cells transfected with 200 ng pCMX–PPARγ1 were treated with (filled columns) or without (open columns) pioglitazone (Pio; 3 μmol/l, 48 h). Data represent mean±S.E.M. ($n=4$), percent of CaMKI without Pio group, normalized by β-actin mRNA levels. ***$P<0{\cdot}001$. (B) Effect of Pio on CaMKI constitutively active form-mediated *CYP11B2* transcriptional activity. H295R cells were transfected with −1521/+2-luc, pCMV-β-gal, pCMX–PPARγ, pcDNA3 (*CaMKI-295* (−)), or CaMKI-295–pcDNA3 (*CaMKI-295* (+)). Cells were treated with Pio (3 μmol/l, 48 h). Data represent mean±S.E.M. ($n=4$), percent of control. ***$P<0{\cdot}001$ versus CaMKI-295 (−). †$P<0{\cdot}001$ versus CaMKI-295 (+). (C) Effect of CaMK inhibitor KN-93 on Pio-mediated *CYP11B2* transrepression. H295R cells were transfected with −1521/+2-luc, pCMV-β-gal, and pCMX–PPARγ1. Cells were treated with Pio (3 μmol/l, 48 h), AII (100 nmol/l, 4 h), or KCl (10·4 mmol/l, 16 mEq/l of K^+, 8 h). KN-93 was added to the media at 5 μmol/l 30 min prior to AII or KCl addition. Data represent mean±S.E.M. ($n=4$), percent of control. ***$P<0{\cdot}001$ versus control. †$P<0{\cdot}001$ versus AII or KCl.

Overexpression of PPARγ reduced the AII-induced *CYP11B2* transcriptional activity as well as enhanced the suppressive effect of pioglitazone (Fig. 2A). Additionally, other PPARγ agonists rosiglitazone and GW1992 also suppressed the AII-induced *CYP11B2* transcriptional activity (Fig. 2B). The effect of pioglitazone on *CYP11B2* transcriptional activity was canceled by PPARγ antagonist GW9662 (Fig. 2C). These data therefore indicate that the pioglitazone-mediated suppression of aldosterone secretion and *CYP11B2* expression are dependent on PPARγ.

CaMKI is reported to be abundantly expressed in the human adrenal zona glomerulosa, and stimulate *CYP11B2* transcriptional activity via the Ad1/CRE element in H295R cells more potently than CaMKIV (Condon *et al.* 2002). Additionally, we demonstrated in this study that the expression level of CaMKI in H295R cells was much higher than that of CaMKIV (Fig. 6A). Therefore, CaMKI is most likely a very important regulator for the Ad1/CRE element in the *CYP11B2* promoter. The Ad1/CRE element in the *CYP11B2* 5′-flanking region is bound by cAMP

response-element-binding protein (CREB), activating transcriptional factor (ATF) 1, and ATF2 (Bassett *et al.* 2000). Interestingly, CREB and ATF1 are reported to be stimulated by CaMKI (Sun *et al.* 1996). Additionally, CREB is known to cooperate with coactivators including steroid receptor coactivator-1 (SRC-1) and p300/CREB-binding protein (CBP) in the AII-induced gene expression (Sahar *et al.* 2007). Moreover, the pioglitazone-mediated *CYP11B2* transrepression was canceled by PPARγ1 L466A/E469A mutant (Fig. 2D) whose function for the ligand-dependent recruitment of coactivators including SRC-1 and CBP is impaired (Gurnell *et al.* 2000). Therefore, these coactivators may be involved in the PPARγ-mediated suppression of *CYP11B2* expression.

The orphan nuclear receptors NGFIB, NURR1, and steroidogenic factor-1 (SF-1) bind to the Ad5 element in the *CYP11B2* 5′-flanking region. NGFIB and NURR1 are known to be upregulated by AII or KCl and positively regulate *CYP11B2* expression, while SF-1 negatively regulates *CYP11B2* expression (Bassett *et al.* 2002). Since the AII/KCl-mediated upregulation of NURR1 is mediated via CaMK, the Ad5 element may possibly be influenced by CaMK (Bassett *et al.* 2004). However, the AII-induced NGFIB and NURR1 expressions were not suppressed by pioglitazone (Supplementary Figure 1), and the pioglitazone-mediated transrepression was not canceled by mutation of the Ad5 element in *CYP11B2* 5′-flanking region (Fig. 4C). Therefore, the Ad5 element may not contribute to the pioglitazone-mediated *CYP11B2* transrepression.

PPARγ1 L466A/E469A double mutant (L468A/E471A in humans) is known to act similarly to PPARγ1 L466A single mutant (Park *et al.* 2003), but that in knockin mice develops hypertension (Freedman *et al.* 2005). Additionally, the PPARγ1 P467L natural mutant in the AF-2 domain (P465L in mice) induces insulin resistance and hypertension in humans (Barroso *et al.* 1999). Since mice expressing human PPARγ1 P467L mutant in VSMCs demonstrate vascular constriction and hypertension (Halabi *et al.* 2008), direct vascular effect may contribute to the progression of hypertension in this mutant. In the presence of PPARγ1 L466A/E469A mutant, impairment of ligand-mediated suppression of aldosterone secretion was observed (Fig. 2E). Therefore, dysregulation of aldosterone production may be involved in the etiology of the PPARγ mutant-induced hypertension.

HX630 augmented the pioglitazone-mediated *CYP11B2* transrepression (Fig. 2F). PPARγ heterodimerizes with RXR (Gearing *et al.* 1993), and binds to and activates PPAR-response elements (PPRE) in the promoter of target genes (IJpenberg *et al.* 1997). However, the Ad1/CRE element, the responsive element of the pioglitazone-mediated *CYP11B2* transrepression, does not contain any consensus PPRE referred to as direct repeat 1 element. Therefore, PPARγ may suppress *CYP11B2* transcriptional activity as a heterodimer with RXR without direct biding to the Ad1/CRE element.

In this study, we used pioglitazone at concentrations of 3 or 10 μmol/l. The experimental concentrations of TZDs are above 10 μmol/l, but TZDs exert some effects, which are PPARγ-independent in NIC-h295 cells at high concentrations (10 μmol/l; Betz *et al.* 2005). Plasma concentration of pioglitazone reaches approximately to 4 μmol/l by 30 mg oral administration in healthy male humans (Takeda Pharmaceuticals, Osaka, Japan). We tested the effects of pioglitazone at 10 μmol/l on aldosterone secretion and *CYP11B2* gene regulation that may include both PPARγ-dependent and -independent effects (Figs 1 and 2A). Therefore, we used pioglitazone at 3 μmol/l, which is similar to the human plasma concentration after oral administration, to exclude PPARγ-independent effects of pioglitazone in most experiments. However, in Fig. 2F, we treated the cells with pioglitazone at 10 μmol/l for 24 h to compare with HX630 at the same pharmacological conditions.

Pioglitazone potentially increased dbcAMP-induced *CYP11B2* transcriptional activity and mRNA expression (Fig. 3C and D). Pioglitazone may increase the dbcAMP-mediated activation of CREB/ATF1, and further studies are needed to be examined.

In conclusion, our observation that PPARγ suppresses *CYP11B2* expression/aldosterone secretion through CaMK may account for the suppressive effects of PPARγ on vascular events associated with atherosclerosis and hypertension.

Supplementary data

This is linked to the online version of the paper at http://dx.doi.org/10.1677/JME-10-0088.

Declaration of interest

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

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