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The effect of pioglitazone on aldosterone and cortisol production in HAC15 human adrenocortical carcinoma cells

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Abstract

Pioglitazone belongs to the class of drugs called thiazolidinediones (TZDs), which are widely used as insulin sensitizers in the treatment of diabetes. A major side effect of TZDs is fluid retention. The steroid hormone aldosterone also promotes sodium and fluid retention; however, the effect of pioglitazone on aldosterone production is controversial. We analyzed the effect of pioglitazone alone and in combination with angiotensin II (AngII) on the late rate-limiting step of adrenocortical steroidogenesis in human adrenocortical carcinoma HAC15 cells. Treatment with pioglitazone for 24hr significantly increased the expression of CYP11B2 and enhanced AngII-induced CYP11B2 expression. Despite the observed changes in mRNA levels, pioglitazone significantly inhibited AngII-induced aldosterone production and CYP11B2 protein levels. On the other hand, pioglitazone stimulated the expression of the unfolded protein response (UPR) marker DDIT3, with this effect occurring at early times and inhibitable by the PPAR γ antagonist GW9962. The levels of DDIT3 (CHOP) and phospho-eIF2 α (Ser51), a UPR-induced event that inhibits protein translation, were also increased. Thus, pioglitazone promotes CYP11B2 expression but nevertheless inhibits aldosterone production in AngII-treated HAC15 cells, likely by blocking global protein translation initiation through DDIT3 and phospho-eIF2 α . In contrast, pioglitazone promoted AngII-induced CYP11B1 expression and cortisol production. Since cortisol enhances lipolysis, this result suggests the possibility that PPARs, activated by products of fatty acid oxidation, stimulate cortisol secretion to promote utilization of fatty acids during fasting. In turn, the ability of pioglitazone to stimulate cortisol production could potentially underlie the effects of this drug on fluid retention.

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Keywords

aldosterone; cortisol; DDIT3; eIF2 α ; endoplasmic reticulum stress; pioglitazone; PPAR; unfolded protein response; zona fasciculata; zona glomerulosa

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Introduction

Aldosterone, the major mineralocorticoid secreted by the glomerulosa cells of the adrenal cortex, is important to maintain fluid and electrolyte balance in vertebrate organisms. Aldosterone production is under the control of circulating angiotensin II (AngII) and potassium (K⁺) levels, as well as adrenocorticotrophic hormone (ACTH) [1]. However, the complex mechanisms that control this process and the interactions between agents that stimulate and inhibit aldosterone production are not well understood. The synthesis of aldosterone requires a chronic rate-limiting step that reflects increased expression of the late regulatory enzyme, aldosterone synthase (CYP11B2) [2].

Thiazolidinediones (TZDs), such as rosiglitazone, pioglitazone, and troglitazone, are a class of antidiabetic drugs that increase insulin sensitivity by directly binding and activating peroxisome proliferator-activated receptor- γ (PPAR γ), a member of the nuclear hormone receptor superfamily of transcription factors. These agents, through PPAR γ , can also stimulate adipocyte differentiation [3–6]. One potential side effect of administration of TZDs is edema (reviewed in [7]), suggesting possible effects of these agents on fluid balance; however, reports concerning pioglitazone effects on aldosterone levels *in vivo* are controversial. Thus, some investigators have reported no effect of TZDs on serum aldosterone levels [8,9], whereas others have reported that these drugs decrease serum aldosterone levels [10]. Still other groups report a trend toward increased serum aldosterone levels and a significant increase in the plasma renin/aldosterone ratio with pioglitazone treatment [11]. This discrepancy may relate in part to the fact that TZDs may have both direct and indirect effects on aldosterone production. Indeed, Uruno et al. [12] have reported that in the human adrenocortical carcinoma cell line H295R, pioglitazone inhibits aldosterone production by inhibiting CYP11B2 expression/promoter activity. On the other hand, pioglitazone reduces serum lipids, including serum triglycerides, a marker of very low density lipoprotein (VLDL) levels, which are known to stimulate aldosterone production *in vitro* [13], suggesting that any beneficial effect of pioglitazone on aldosterone levels may occur through its improvement of elevated VLDL levels. In addition, pioglitazone can activate PPAR α in addition to PPAR γ [14], and in fact, this lack of PPAR γ specificity appears to improve its safety profile relative to the more selective PPAR γ agonists such as rosiglitazone [15]. Therefore, the physiological role of PPAR γ in adrenocortical cells remains largely unclear, indicating the importance of understanding the effects of pioglitazone on aldosterone production.

We investigated the possible role of PPARs and the agonist pioglitazone in human adrenocortical HAC15 cells, a clone of NCI H295R cells [16,17]. HAC15 cells were chosen because these cells represent an established model to study steroidogenesis in the human adrenal cortex. They produce large amounts of aldosterone; nevertheless, these cells are

dedifferentiated, express the genes that encode the steroidogenic enzymes present in all three layers of the adult adrenal cortex and can be induced to produce all of the steroid hormones produced by the adrenal cortex [18]. In this study, we investigated the regulation of human genes involved in aldosterone biosynthesis using pioglitazone and AngII.

Materials and Methods

Chemicals and antibodies

AngII, pioglitazone and GW9662 were purchased from Sigma (St. Louis, MO, USA). DMEM/F12 (1:1) medium was purchased from Gibco (Invitrogen Life Technologies, Grand Island, NY, USA). Cosmic calf serum (CCS) was obtained from Hyclone Thermo Fisher Scientific (Waltham, MA). Penicillin-streptomycin was purchased from Gibco, and gentamicin was obtained from Invitrogen. ITS+ Premix Universal Culture Supplement was purchased from BD Biosciences (Franklin Lakes, NJ). Trypsin-EDTA (0.05%) was obtained from Life Technologies, GW6471 from Tocris Biosciences (Minneapolis, MN) and tauroursodeoxycholic acid (TUDCA) from EMD Millipore (Billerica, MA). The rabbit anti-DDIT3/CHOP/GADD153 antibody and mouse anti-GAPDH antibody were obtained from Novus Biologicals (Littleton, CO, USA), the rabbit anti-phospho-eIF2 α (Ser51) antibody was obtained from Cell Signaling Technology (Danvers, MA, USA), the rabbit anti-StAR was purchased from Abcam (Cambridge, MA, USA) and the mouse anti- β -actin antibody was obtained from Sigma. The mouse anti-CYP11B2 antibody was a generous gift from Dr. Celso Gomez-Sanchez (University of Mississippi Medical Center, Jackson, MS). The goat anti-rabbit and goat anti-mouse secondary antibodies were from LI-COR Biosciences (Lincoln, NE, USA). Coat-A-Count aldosterone assay kits were purchased from Siemens (Munich, Germany) and the cortisol EIA assay kit from Oxford Biomedical Research (Rochester Hills, MI).

Cell culture and treatment

The human adrenocortical carcinoma HAC15 cell line [18] which is a clone of the H295R cell line [16,17], was kindly provided by Dr. William Rainey (University of Michigan, Ann Arbor, MI) and was grown in DMEM/F12 medium containing 1% ITS, 1% penicillin–streptomycin, 0.01% gentamicin and 10% CCS. For analysis of responses to AngII and/or pioglitazone, cells were sub-cultured in 6-well plates to approximately 75–80% confluence. One day before the experiment, the medium was replaced with a low-serum experimental medium (DMEM/F12 medium supplemented with 0.1% CCS). The next morning, cells were treated with 0.1% DMSO or 10 μ M pioglitazone in the presence and absence of 10nM AngII in fresh low-serum experimental medium. For the dose response experiments, the supernatants were collected and cells were harvested after treatment for 24hr with 300nM, 1 μ M, 3 μ M and 10 μ M pioglitazone. The vehicle DMSO (0.1%) was used as the control group in all experiments. For the time course experiments, the supernatants were collected and cells were harvested after treatment with 10 μ M pioglitazone for 4, 8, 12, 16, 24 or 36hr as indicated. For experiments examining StAR expression, cells were treated in two different ways: in one set of experiments cells were treated for 1hr with or without 10nM AngII in the presence or absence of 10 μ M pioglitazone; in the other, the cells were pretreated for 24hr with DMSO or 10 μ M pioglitazone, followed by treatment with or

without 10nM AngII for 1hr. For some experiments, cells were treated with 10 μ M pioglitazone and 10 μ M GW9662 or GW6471 or 500 μ M TUDCA for 24hr.

Measurement of steroid hormone production

HAC15 cells were incubated for 16–20hr in low-serum DMEM/F12 medium containing 0.1% CCS. The cells were treated with and without AngII and/or pioglitazone for the times indicated. The supernatants were collected and stored frozen at –20°C until aldosterone was assayed using Coat-A-Count RIA kits (Siemens). The radioactivity was measured by a multicrystal γ -counter (Berthold Technologies, Bad Wildbad, Germany) as described [19]. Cortisol was measured by an Oxford Biomedical Research cortisol EIA kit according to the manufacturer's instructions. Results of aldosterone and cortisol assays were normalized to the amount of cellular protein and expressed as pmol per milligram of cell protein.

RNA isolation, cDNA synthesis and real-time quantitative RT-PCR (qRT-PCR)

Total RNA was extracted using the RNeasy plus Mini Kit (Qiagen, Valencia, CA, USA) or TRIzol (Invitrogen) according to protocols from the manufacturers. Purity and integrity of the RNA were checked spectroscopically using a NanoDrop 2000/c spectrophotometer (Thermo, USA). Then, for each sample, 2 μ g RNA was reverse transcribed to obtain the cDNA template using the high-capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA, USA) following the manufacturer's protocol. Each cDNA sample was diluted 5 times for real-time quantitative RT-PCR (qRT-PCR) amplification; qRT-PCR was performed using the Applied Biosystems StepOne and StepOnePlus Real-Time PCR Systems. Amplification was performed with the following fast time course: 95°C 20s, 95°C 1s, 65°C for 20s for 40 cycles.

Relative mRNA expression values were determined by the 2^{-C_t} method using human cyclophilin (PPIA) as the normalization control. The cycle-to-threshold (C_t) values were calculated for statistical analysis. Correction for loading control, C_t , is calculated as $C_{t_{\text{target}}} - C_{t_{\text{reference}}}$, and reference is the C_t value for PPIA; C_t is expressed as $C_{t_{\text{treated}}} - C_{t_{\text{control}}}$. Fold change in mRNA expression of each sample by treatment is then calculated by the formula 2^{-C_t} [20]. The probe sets for human CYP11B2 (Hs01597732_m1), CYP11B1 (Hs01596404_m1), CYP17A1 (Hs01124136_m1), CYP21A2 (Hs00416901_g1), DDIT3 (Hs00358796_g1), StAR (Hs00986559_g1) and PPIA (Hs04194521_s1) were purchased from Applied Biosystems (Supplemental Table 1).

Western blot analysis

Cells incubated for 16–20hr in 0.1% low-serum medium were treated with the appropriate agents as indicated. Cells were lysed with SDS lysis buffer (0.1875M Tris-HCl, 3% SDS and 1.5mM EGTA). The cell lysates were centrifuged and the supernatants mixed with 3 $\times$ Sample buffer (30% glycerol, 15% 2-mercaptoethanol and 1% bromophenol blue). The proteins were separated through 10% mini-SDS-PAGE gels and electrophoretically transferred to PVDF membranes. The membranes were then blocked by incubating in the blocking buffer (5% nonfat dry milk, 150mM NaCl, 10mM Tris-HCl, pH 7.5) for 1hr and then incubated with blocking buffer containing DDIT3/CHOP/GADD153 antibody (1:1000), phospho-eIF2 α (Ser51) antibody (1:500), StAR antibody (1:15,000), anti- β -actin

antibody (1:15,000) or anti-GAPDH antibody (1:10,000) overnight at 4°C. Following extensive washing in Tris-buffered saline containing 0.1% Tween-20 (TBST) buffer, the transfer membranes were further incubated for 1.5hr in 5% nonfat dry milk blotting buffer that contained IRDye-conjugated secondary antibodies. Then, the membranes were washed three times with TBST. Finally, an Odyssey imaging system (Licor Biosciences, Lincoln, NE) was utilized to visualize and quantify immunoreactivity, using Odyssey application software (version 2.1). Proteins were normalized to β -actin or GAPDH as indicated.

Statistical analysis

All experiments were repeated a minimum of three times and the values obtained expressed as the means $\pm$ SEM. Significant differences were accepted at the 0.05 level of probability and were statistically determined with ANOVA followed by a Newman-Keuls post-hoc test using GraphPad Prism4 (San Diego, CA).

Results

Changes in CYP11B2 expression in response to pioglitazone alone and in the presence of AngII

Uruno et al. [12] have previously shown that pioglitazone suppresses the AngII-induced expression of CYP11B2, largely with the use of a construct in which an approximately 1.5 kbase CYP11B2 promoter fragment drives expression of a luciferase reporter. The CYP11B2 gene encodes the enzyme aldosterone synthase catalyzing the final reactions in aldosterone biosynthesis and is the late rate-limiting step in aldosterone production. We sought to verify this result in HAC15 cells derived from the H295R cell line [16,17]. Unexpectedly, we observed that pioglitazone significantly enhanced the ability of AngII to stimulate CYP11B2 expression (Figure 1A). We considered the possibility that our disparate results related to differences in the primer/probe set used, but when we performed qRT-PCR with the identical primer/probe set used by Uruno et al., we nevertheless found that pioglitazone again tended to enhance the ability of AngII to stimulate CYP11B2 expression (Figure 1B).

Pioglitazone increased CYP11B2 expression in a time- and dose-dependent manner

We then studied the dose dependence of the effects of pioglitazone on CYP11B2 gene expression. Using doses ranging from 0.3 to 10 μ M, we found that pioglitazone stimulated the expression of CYP11B2 in a dose-dependent manner. CYP11B2 expression was up-regulated by increasing doses of pioglitazone with a significant increase observed in response to a 10 μ M concentration (Figure 2A). Concentrations of 3–30 μ M have been reported to overlap the concentrations of the drug in humans [21]. We then investigated the time dependence of the effect of pioglitazone on HAC15 cells. First, we studied a longer time course and observed that CYP11B2 expression was significantly enhanced at 16hr, and steadily increased to more than 20 times the control group after treatment for 36hr (Figure 2B). We also examined a shorter time course for CYP11B2 gene expression and found that there were no differences upon treatment for 4, 8, or 12hr (data not shown).

Pioglitazone inhibited AngII-induced CYP11B2 protein expression and aldosterone production

We next determined whether the increase in CYP11B2 expression was translated into enhanced protein levels. In contrast to the significant increase in CYP11B2 expression seen with pioglitazone treatment, this agent inhibited the AngII-induced increase in CYP11B2 levels (Figure 3A and B). The ability of pioglitazone to inhibit AngII-stimulated CYP11B2 protein expression suggested that this agent would inhibit AngII-induced aldosterone production, despite its ability to induce CYP11B2 mRNA expression. Indeed, 10nM AngII treatment for either 24hr or 48hr significantly increased aldosterone production in HAC15 cells, and pioglitazone inhibited this steroidogenic response to AngII (Figure 3C and D). In addition, at earlier times pioglitazone alone slightly but significantly inhibited basal aldosterone production (Figure 3E), although the TZD had no effect on aldosterone production at later times (Supplemental Figure 1). This result indicates that despite the increase in CYP11B2 expression, pioglitazone somehow suppressed aldosterone production. We considered the possibility that aldosterone production might be decreased by pioglitazone because of an ability of the TZD to induce CYP17A1, thereby promoting cortisol or adrenal androgen biosynthesis at the expense of aldosterone production. However, our results demonstrated that pioglitazone actually slightly inhibited CYP17A1 expression, alone and in combination with AngII (Supplemental Figure 2).

Pioglitazone pretreatment stimulates StAR mRNA and protein expression in HAC15 cells

We also considered the possibility that pioglitazone inhibited the expression of StAR, a key gene product required for the synthesis of all steroid hormones. StAR is responsible for the early rate-limiting transport of cholesterol from the outer to the inner mitochondrial membrane, at which site the initial enzymatic reaction, cholesterol side chain cleavage, is catalyzed. We, therefore, investigated the effect of pioglitazone, alone and in combination with AngII exposure, on StAR expression after an acute 1hr stimulation. Pioglitazone had no effect at this short time point either in the presence or absence of AngII (Supplemental Figure 3). In contrast, a 24hr pretreatment with pioglitazone significantly induced StAR gene expression alone and enhanced the stimulatory response to AngII (Figure 4A). To determine whether the changes in StAR expression were translated into alterations in protein levels and could therefore affect steroidogenesis, cells were treated with or without 10nM AngII and/or 10 μ M pioglitazone for 6hr, and StAR protein expression was detected by western blot. StAR protein levels were increased significantly by AngII or pioglitazone or the combination (Figure 4B). This result predicts that more cholesterol can be transported into the inner mitochondrial membrane for the early stage of steroidogenesis and suggests that the effect of pioglitazone to inhibit aldosterone synthesis is not related to an ability to alter StAR expression/levels.

Pioglitazone increased DDIT3 mRNA and protein expression and eIF2 α phosphorylation

In a microarray study in HAC15 cells, DNA damage-induced transcript 3 (DDIT3) was one of the top pioglitazone up-regulated genes (data not shown). DDIT3 encodes the protein C/EBP homologous protein (CHOP), also known as G1 arrest and DNA damage 153 (GADD153), which is a transcription factor known to be induced by endoplasmic reticulum

(ER) stress. Upon the induction of ER stress, multiple pathways are activated to initiate the unfolded protein response (UPR), in an attempt to ameliorate the stress; one such pathway results in activation of the kinase, double-stranded RNA-dependent protein kinase-like ER kinase (PERK) to trigger eukaryotic initiation factor 2 α (eIF2 α) phosphorylation. Phosphorylated eIF2 α subsequently attenuates the rate of global translation to prevent further protein synthesis and reduce the ER protein load (reviewed in [22]). We hypothesized that ER stress and induction of the UPR might be involved in the inability to translate the increased CYP11B2 mRNA levels into protein.

Therefore, we first verified the effect of pioglitazone on DDIT3 expression by qRT-PCR. As shown in Figure 5A, pioglitazone increased DDIT3 expression, and there was no significant difference between pioglitazone alone and in combination with AngII. A dose response experiment was performed and demonstrated that only 10 μ M pioglitazone induced an increase in DDIT3 expression (Figure 5B). This pioglitazone-induced DDIT3 expression occurred in a time-dependent manner, with pioglitazone enhancing DDIT3 expression both at early and late time points. DDIT3 expression was significantly increased after 24hr (Figure 5D). Pioglitazone also significantly increased DDIT3 expression within 4hr of exposure (Figure 5C). Although DDIT3 expression for the 12hr time point of Figure 5C was significantly different from the control value whereas the same time point in Figure 5D was not, this anomaly was the result of the high values observed at the 36hr time point. When the 36hr time point was excluded from the statistical analysis of the data in Figure 5D, the 12hr, 16hr, 24hr time point were found to be significantly increased compared to control. To verify that ER stress could induce DDIT3 expression in HAC15 cells, we also showed that the ER stress inducers thapsigargin (TG) and tunicamycin (TUN) stimulated DDIT3 gene expression (Supplemental Figure 4). Finally, we demonstrated that the DDIT3 mRNA expression was translated into protein. Thus, western analysis showed that pioglitazone increased DDIT3 (CHOP) levels and this increase was not affected by costimulation with AngII (Figure 6A). AngII alone also enhanced DDIT3 (CHOP) protein expression (Figure 6A). The levels of phospho-eIF2 α (Ser51) were also significantly increased by pioglitazone alone or in combination with AngII (Figure 6B). This result suggests that pioglitazone may trigger the UPR, which could attenuate CYP11B2 translation to result in a reduction in aldosterone production.

The induction of DDIT3 occurs through PPAR γ while that of CYP11B2 occurs through both PPAR α (and possibly PPAR γ) as well as ER stress/UPR

To determine the mechanism by which pioglitazone induced the expression of CYP11B2 and DDIT3, cells were stimulated with or without pioglitazone in the presence or absence of antagonists selective for PPAR γ or PPAR α , GW9662 [23] and GW6471 [24], respectively. As shown in Figure 7A, pioglitazone-induced DDIT3 expression was inhibited by GW9662, suggesting that this effect was mediated through PPAR γ . On the other hand, incubation with GW6471 alone stimulated DDIT3 expression, and had no effect on pioglitazone-induced DDIT3 mRNA levels (Supplemental Figure 5). In contrast, GW6471 inhibited pioglitazone-induced CYP11B2 expression, suggesting the involvement of PPAR α in this process (Figure 7B). On the other hand, the PPAR γ -selective antagonist GW9662 also inhibited pioglitazone-induced expression of CYP11B2, as well as CYP11B1 (Supplemental Figure

6). Whether this inhibition reflects a role for PPAR γ in the induction of expression of these genes or is the result of some lack of selectivity of the antagonist is unclear. However, it should be noted that GW9962 is reported to inhibit ligand binding to PPAR α , albeit at an approximately 10-fold lesser potency [23]. In addition, incubation of the cells with tauroursodeoxycholic acid (TUDCA), a chemical chaperone known to ameliorate ER stress [25], inhibited the increase in CYP11B2 gene expression (Figure 7D) but not that of DDIT3 (Figure 7C). Together with the time course of DDIT3 expression, these results suggest that the induction of DDIT3 is via effects on PPAR γ -mediated gene transcription, whereas that of CYP11B2 may occur through both PPAR α and the UPR (and possibly PPAR γ).

Pioglitazone stimulated CYP11B1 expression alone and in combination with AngII

HAC15 cells are known to produce all of the steroid hormones synthesized in the adrenal cortex. Previously, Uruno et al. [12] did not examine the effect of pioglitazone on the expression of other genes encoding important steroidogenic enzymes or on the synthesis of other steroid hormones, such as cortisol. Cortisol is a stress hormone known to regulate lipid metabolism, as do the PPARs (reviewed in [26–28]). Therefore, we examined the effect of pioglitazone on the expression of the gene encoding 11 β -hydroxylase, the key enzyme in cortisol biosynthesis, which is encoded by the CYP11B1 gene. Our results showed that CYP11B1 expression was significantly up-regulated by AngII, and this stimulatory effect was enhanced by pioglitazone (Figure 8A). This effect was time- and dose-dependent (Figure 8B and C), with maximal CYP11B1 observed at 10 μ M pioglitazone and 24hr of stimulation. Incubation with pioglitazone for periods of time less than 12hr had no effect on CYP11B1 expression (data not shown). As with CYP11B2, inhibition of PPAR α with GW6471 completely blocked pioglitazone-induced CYP11B1 expression (Figure 8D). Thus, the data suggest that pioglitazone, alone and in combination with AngII, can increase the expression of the gene encoding the final enzymatic step in cortisol production, 11 β -hydroxylase, through activation of PPAR α .

Pioglitazone stimulated cortisol production alone and in combination with AngII

Finally, we also measured cortisol production after a 24hr treatment with or without pioglitazone in the presence and absence of AngII. Consistent with its effects on CYP11B1 expression, pioglitazone significantly increased cortisol release into the medium, as did AngII alone, and the combination enhanced cortisol synthesis further (Figure 9). On the other hand, pioglitazone had no effect, either alone or in combination with AngII, on the expression of CYP21A2, a steroidogenic enzyme that would tend to also promote cortisol production, whereas AngII treatment enhanced its expression (Supplemental Figure 7).

Discussion

In this study we analyzed the mechanisms by which pioglitazone decreased the biosynthesis of aldosterone in human adrenocortical cells despite an ability of the TZD to increase the mRNA expression of the key late rate-limiting biosynthetic enzyme, aldosterone synthase or CYP11B2. However, although mRNA expression of CYP11B2 was increased by pioglitazone, AngII's induction of protein expression was inhibited by this agent. The mechanism may involve the unfolded protein response, also known as ER stress, which has

been found to modulate translation through eIF-2 α (25), as pioglitazone was observed to induce DDIT3 (also known as CHOP) mRNA and protein expression. Indeed, pioglitazone induced phosphorylation of eIF-2 α at serine 51, a post-translational modification that represses the initiation step of protein synthesis [29,30]. Alternatively, DDIT3 is known to be proapoptotic and could be affecting CYP11B2 levels through caspase-mediated degradation, in which case the increased mRNA levels might represent an accompanying compensatory mechanism. In any case, the present data demonstrate that pioglitazone was able to reduce aldosterone production in response to AngII in steroidogenic human adrenocortical carcinoma (HAC15) cells.

It is known that pioglitazone has high affinity for the nuclear receptor PPAR γ , and this drug is used clinically to treat type II diabetes. PPAR γ agonists have been reported to lower blood pressure and provide other cardiovascular benefits, and PPAR γ agonists suppress AngII type 1 receptor expression [31]. In addition, pioglitazone attenuates AngII-induced cardiac fibrosis by inhibiting myocardial macrophage infiltration and pro-inflammatory gene expression [32]. However, some investigators report that pioglitazone does not affect the circulating levels of aldosterone, renin activity or AngII in diabetic patients [9,33]. Similarly, PPAR γ agonists such as pioglitazone and rosiglitazone are reported to have no effect on AngII-induced plasma aldosterone levels in Sprague–Dawley rats [34]. However, PPAR γ agonists inhibit aldosterone-induced mesangial cell proliferation [35] and significantly reduce plasma aldosterone levels in renal collecting duct-specific PPAR γ knockout mice [36]. Thus, the effect of PPAR γ agonists on serum aldosterone levels remains somewhat controversial. Nevertheless, pioglitazone also exhibits agonist activity for PPAR α [14]. Our results showing that the PPAR α antagonist GW6471 inhibited pioglitazone-enhanced CYP11B2 and CYP11B1 expression suggest the likely importance of pioglitazone's effect on PPAR α . Conversely, the PPAR γ antagonist GW9662 inhibited pioglitazone-induced DDIT3 expression (Figure 6). However, surprisingly, GW6471 significantly increased DDIT3 gene expression as well and this effect could be partially inhibited by GW9662 (data not shown), similarly to the effect of pioglitazone (Supplemental Figure 4), suggesting that these antagonists are not entirely selective. Reported effects of pioglitazone on PPAR β/δ [37] could possibly partially explain these somewhat anomalous data.

It should be noted that our results demonstrating that pioglitazone increased CYP11B2 mRNA expression, alone and in AngII-stimulated cells, are in contrast to the results of Uruno et al. [12], who observed that pioglitazone inhibited AngII-induced CYP11B2 by a direct effect on transcription. The reason for this disparity is not clear, although we were concerned that the difference could be the result of the dissimilar primer/probe sets used for the qRT-PCR analysis. However, using the same primer/probe set as Uruno et al. [12], which recognized a sequence in the 3'-UTR of CYP11B2, we observed that a 24hr treatment with pioglitazone still increased CYP11B2 expression (Fig. 1B). Another difference between the two studies is the length of time of pioglitazone treatment. In the Uruno et al. report, the H295R cells were treated for 72hr prior to measurement of CYP11B2 mRNA expression [12], whereas in our experiments the HAC15 cells were treated in general for 24hr (Figure 2). It is possible that there may be feedback (either positive or negative) with longer exposures. Moreover, Uruno et al. [12] failed to show the effect of pioglitazone

alone. Also, these authors utilized H295R cells as a model, while we used the HAC15 cell line, which exhibits a greater ability to secrete aldosterone; it seems possible that the difference in the model system could potentially underlie the disparate results, although this seems unlikely since the HAC15 cell line is a clone of the H295R cells [16,17]. Finally, the majority of the data were obtained by Uruno et al. [12] using an exogenous CYP11B2 promoter reporter construct transfected into H295R cells, whereby it was determined that pioglitazone inhibited CYP11B2 expression through direct PPAR γ -mediated effects on transcription. The construct used in these studies may lack regulatory elements upstream of the approximately 1.5kb promoter used, as well as any elements present in introns or in the 3'-UTR, such that regulation of the reporter construct may not be identical to that of the endogenous gene. Indeed, we observed that treatment of HAC15 cells with the protein synthesis inhibitor cycloheximide prevented the pioglitazone-induced increase in CYP11B2 mRNA levels, but not those of DDIT3 (unpublished observations). This result suggests that in HAC15 cells, CYP11B2 is not a direct target of pioglitazone/PPAR γ .

The PPARs are involved in lipid homeostasis with some isoforms activated under conditions of lipid excess to promote fat storage (PPAR γ) and others activated by products of lipid metabolism upon nutrient deprivation to promote the use of fat for energy (PPAR α) (reviewed in [26,27]). Cortisol is a steroid hormone produced and secreted by the zona fasciculata of the adrenal cortex during times of stress, such as fasting, and assists in the response to this stress. Thus, one of the effects of cortisol is to enhance lipid metabolism through binding to the glucocorticoid receptor. Therefore, it seemed possible that activation of PPARs might modulate cortisol production in the adrenal cortex. Using the PPAR α / γ agonist pioglitazone in the dedifferentiated adrenocortical carcinoma HAC15 cell line, we found that this agent increased the expression of CYP11B1, a key cortisol synthetic enzyme through activation of PPAR α (Figure 7), as well as the production of cortisol in these cells (Figure 8). This effect of pioglitazone was also likely due in part to its ability to stimulate StAR mRNA and protein levels (Figure 4). Interestingly, StAR gene expression was not changed by AngII or pioglitazone after a 1hr treatment; however, StAR protein expression was increased significantly by AngII or pioglitazone treatment for 6hr. Moreover, in HAC15 cells pretreated with pioglitazone for 24hr, the expression of StAR was increased. Elevated StAR mRNA and protein levels (Figure 4) should lead to enhanced adrenocortical steroidogenesis, with production of cortisol (Figure 9) as a result of the increased CYP11B1 expression (Figure 8), the maintained CYP21A2 (Supplemental Figure 7) and decreased CYP11B2 levels (Figure 3).

In addition to its effects on lipid metabolism, cortisol also may have effects on fluid retention through its ability to bind to the mineralocorticoid receptor (MR). In many tissues the MR is protected from cortisol, which otherwise binds with high affinity to the MR, by the presence of the cortisol-inactivating enzyme 11 β -hydroxysteroid dehydrogenase-2 (11 β -HSD2). However, PPARs have been found to inhibit 11 β -HSD2 expression in some cell types and induce the cortisol-activating enzyme 11 β -hydroxysteroid dehydrogenase-1 (11 β -HSD1) in others [38,39]. Indeed, cortisol is known to cause hypertension under certain conditions and has been postulated to play a role in the metabolic syndrome (reviewed in [40]). Therefore, it seems possible that the effect of pioglitazone on cortisol production

could contribute to the known adverse side effect of fluid retention observed with the use of the drug.

In summary, our results show that pioglitazone, alone and in combination with AngII, enhances the expression of CYP11B2 in HAC15 adrenocortical carcinoma cells. However, the TZD actually inhibits both CYP11B2 protein expression and aldosterone production, likely through its ability to induce the UPR, increase DDIT3 mRNA and protein expression and stimulate the phosphorylation of eIF2 α , which is known to trigger a global inhibition of translation. On the other hand, phosphorylated eIF2 α can selectively increase the translation of certain mRNAs, such as activating transcription factor 4 (ATF4), with the result that these genes can escape from the eIF2 α -mediated translation attenuation (reviewed in [22]). Indeed, pioglitazone stimulates cortisol production, suggesting that the TZD-increased CYP11B1 mRNA levels are translated to protein. The synthesized cortisol would presumably serve to facilitate lipid metabolism under physiological conditions. In addition, glucocorticoids acting through the glucocorticoid receptor have been demonstrated to increase PPAR α expression in hepatocytes [41], suggesting a possible feed-forward mechanism. This effect of pioglitazone on cortisol may also underlie the side effect of fluid retention observed with TZD use, and our results suggest that the co-administration of pioglitazone with an MR antagonist may improve diabetes therapy while minimizing adverse side effects.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

Pioglitazone inhibits aldosterone production despite increasing CYP11B2 mRNA levels

Pioglitazone causes ER stress, induces DDIT3 and increases eIF2 α phosphorylation

Pioglitazone-inhibited CYP11B2 protein levels are likely due to the induced ER stress

Pioglitazone induces CYP11B1 and StAR expression and enhances cortisol synthesis

Cortisol may cooperate with PPAR activated by lipid metabolites to regulate fat usage

Figure 1. Pioglitazone enhanced the stimulatory effect of AngII on CYP11B2 gene expression
 HAC15 cells were treated with or without 10nM AngII in the presence or absence of 10μM pioglitazone (Pio) for 24hr; 0.1% DMSO (Con) was used as the vehicle. CYP11B2 mRNA expression was detected by qRT-PCR using (A) the commercially available ABI primer/probe set. Values represent the means $\pm$ SEM of six separate experiments performed in duplicate and are expressed relative to the control, normalized to PPIA expression; ** $P < 0.01$ and *** $P < 0.001$ vs control, †† $P < 0.01$ vs AngII. For panel (B), CYP11B2 expression was determined using the primer/probe sets described in Uruno et al. (2011) and obtained from Integrated DNA Technologies, Inc. (Coralville, Iowa, USA). Values represent the means $\pm$ SEM of three separate experiments performed in duplicate and are expressed relative to the control, normalized to PPIA expression; ** $P < 0.01$ and *** $P < 0.001$ versus the control value.

Figure 2. HAC15 cells responded in a dose- and time-dependent manner to pioglitazone
 (A) Cells were treated with doses of 0.3μM, 1μM, 3μM, or 10μM pioglitazone (Pio) for 24hr with 0.1% DMSO as vehicle (Con). CYP11B2 mRNA expression was measured by qRT-PCR. Values represent the means $\pm$ SEM of three separate experiments performed in duplicate and are expressed relative to the control and normalized to PPIA mRNA levels; * P < 0.05 vs control. (B) Cells were treated with 10μM pioglitazone for the indicated times with 0.1% DMSO as vehicle (Con). CYP11B2 mRNA expression was measured by qRT-PCR. Values represent the means $\pm$ SEM of four separate experiments performed in duplicate, expressed relative to the control and normalized by PPIA mRNA levels; * P < 0.05 and *** P < 0.001 vs control.

Figure 3. Pioglitazone inhibited AngII-induced CYP11B2 protein levels and aldosterone production in HAC15 cells

(A) Cells were treated with 0.1% DMSO or 10 μ M pioglitazone (Pio) in the presence and absence of 10nM AngII for 24hr. The cells were collected in 3% SDS lysis buffer, as described in Methods, and proteins were analyzed by western blotting for CYP11B2 protein levels. (B) Cumulative values for CYP11B2 protein levels represent the means $\pm$ SEM of three-five separate experiments performed in duplicate and normalized to the loading control GAPDH; * P < 0.05 and *** P < 0.001 vs control; ††† P < 0.001 vs AngII. (C-E) Cells were treated with 0.1% DMSO or 10 μ M pioglitazone in the presence and absence of 10nM AngII. After the indicated times, the supernatants were collected and aldosterone was assayed by RIA. Results are shown for cells treated with or without 10 μ M pioglitazone and/or 10nM AngII (C) for 24hr and (D) for 48hr. (E) Cells were treated with 10 μ M pioglitazone alone for shorter times as indicated. Values represent the means $\pm$ SEM of three-four separate experiments performed in duplicate; * P < 0.05, ** P < 0.01 and *** P < 0.001 vs control; ††† P < 0.01 vs AngII alone.

Figure 4. Pioglitazone stimulated StAR mRNA and protein expression induced by AngII in HAC15 cells

(A) Cells were pretreated with 0.1% DMSO or 10 μ M pioglitazone (Pio) for 24hr, before treatment with 10nM AngII for 1hr. StAR mRNA expression was detected by qRT-PCR and the results shown. Values represent the means $\pm$ SEM of three separate experiments performed in duplicate normalized to PPIA mRNA levels and expressed relative to the control; * P < 0.05 and *** P < 0.001 vs control; † P < 0.05 vs AngII alone. (B) Cells were treated with 0.1% DMSO or 10 μ M pioglitazone in the presence and absence of 10nM AngII for 6 hours. The cells were collected in 3% SDS lysis buffer, and StAR protein levels were analyzed by western blotting, with a representative blot shown. Cumulative values represent the means $\pm$ SEM of three separate experiments performed in duplicate and normalized to the loading control β -actin: * P < 0.05 and *** P < 0.001 vs control.

Figure 5. Pioglitazone induced DDIT3 gene expression in a dose- and time-dependent manner (A) HAC15 cells were treated with or without 10nM AngII in the presence or absence of 10μM pioglitazone for 24hr; 0.1% DMSO (Con) was used as the vehicle. DDIT3 mRNA expression was detected by qRT-PCR. Values represent the means $\pm$ SEM of six separate experiments performed in duplicate and are expressed relative to the control, normalized to PPIA expression; * $P < 0.05$ vs control, † $P < 0.05$ vs AngII alone. (B) Cells were treated with doses of 0.3μM, 1μM, 3μM, or 10μM pioglitazone (Pio) for 24hr with 0.1% DMSO as vehicle (Con). DDIT3 mRNA expression was measured by qRT-PCR. Values represent the means $\pm$ SEM of three separate experiments performed in duplicate and are expressed relative to the control and normalized to PPIA mRNA levels; ** $P < 0.01$ vs control. (C and D) Cells were treated with 10μM pioglitazone for the indicated times with 0.1% DMSO as vehicle (Con). DDIT3 mRNA expression was measured by qRT-PCR. Values represent the means $\pm$ SEM of four separate experiments performed in duplicate, expressed relative to the control and normalized by PPIA mRNA levels; * $P < 0.05$ and *** $P < 0.001$ vs control.

Figure 7. Pioglitazone induced DDIT3 via PPAR γ and CYP11B2 through PPAR α activation and ER stress

(A) Cells were treated with or without 10 μ M pioglitazone (Pio) in the presence and absence of the PPAR γ antagonist GW9662 and DDIT3 expression monitored by qRT-PCR. (B) Cells were treated with or without 10 μ M pioglitazone and/or the PPAR α antagonist 10 μ M GW6471 and CYP11B2 expression monitored by qRT-PCR. (C and D) Cells were treated with or without 10 μ M pioglitazone in the presence and absence of the ER stress inhibitor TUDCA (500 μ M). (C) DDIT3 or (D) CYP11B2 mRNA levels were then monitored by qRT-PCR. Values represent the means $\pm$ SEM of three-four separate experiments performed in duplicate; ** P < 0.01 and *** P < 0.001 vs control; ff P < 0.01 and fff P < 0.001 vs Pio alone.

Figure 8. Pioglitazone enhanced the stimulatory effect of AngII on CYP11B1 mRNA levels and induced CYP11B1 expression in a dose- and time-dependent manner

(A) HAC15 cells were treated with or without 10nM AngII in the presence or absence of 10μM pioglitazone (Pio) for 24hr; 0.1% DMSO (Con) was used as the vehicle. CYP11B1 mRNA expression was detected by qRT-PCR. Values represent the means $\pm$ SEM of five separate experiments performed in duplicate and are expressed relative to the control, normalized to PPIA expression; * P < 0.05 and *** P < 0.001 vs control, † P < 0.05 vs AngII alone. (B) Cells were treated with doses of 0.3μM, 1μM, 3μM, or 10μM pioglitazone for 24hr with 0.1% DMSO as vehicle (Con). CYP11B1 mRNA expression was measured by qRT-PCR. Values represent the means $\pm$ SEM of three separate experiments performed in duplicate and are expressed relative to the control and normalized to PPIA mRNA levels; * P < 0.05 vs control. (C) Cells were treated with 10μM pioglitazone for the indicated times with 0.1% DMSO as vehicle (Con). CYP11B1 mRNA expression was measured by qRT-PCR. Values represent the means $\pm$ SEM of three separate experiments performed in duplicate and are expressed relative to the control and normalized to PPIA mRNA levels; * P < 0.05, ** P < 0.01 and *** P < 0.001 vs control. (D) Cells were treated with or without 10μM pioglitazone and/or the PPAR α antagonist 10μM GW6471 and CYP11B1 expression monitored by qRT-PCR. Values represent the means $\pm$ SEM of three-four separate experiments performed in duplicate, expressed relative to the control and normalized by PPIA mRNA levels; ** P < 0.01 vs control, ff P < 0.01 vs Pio alone.

Figure 9. Pioglitazone enhanced the effect of AngII to stimulate cortisol production

Cells were treated with 0.1% DMSO or 10 μ M pioglitazone (Pio) in the presence and absence of 10nM AngII. After the indicated times, the supernatants were collected and cortisol was assayed by EIA. Results are shown for cells treated with or without 10 μ M pioglitazone and/or 10nM AngII for 24hr. Values represent the means $\pm$ SEM of three-four separate experiments performed in duplicate; *** P < 0.001 vs control; ††† P < 0.001 vs AngII alone.