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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 24 h 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 PPARγ antagonist GW9962. The levels of DDIT3 (CHOP) and phospho-eIF2α (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-eIF2α. 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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1. 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 adrenocorticotropic hormone (ACTH) (Spat and Hunyady, 2004). 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) (Miller and Auchus, 2011).

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 (PPARγ), a member of the nuclear hormone receptor superfamily of transcription factors. These agents, through PPARγ, can also stimulate adipocyte differentiation (Lehmann et al., 1995; Yki-Jarvinen, 2004; Lehrke and Lazar, 2005; Heikkinen et al., 2007). One potential side effect of administration of TZDs is edema (reviewed in Starner et al., 2008), 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 (Zanchi et al., 2010; Kurisu et al., 2013), whereas others have reported that these drugs decrease

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serum aldosterone levels (Eguchi et al., 2007). Still others report a trend toward increased serum aldosterone levels and a significant increase in the plasma renin/aldosterone ratio with pioglitazone treatment (Zanchi et al., 2004). 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. (2011) 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 (Betteridge, 2007), a marker of very low density lipoprotein (VLDL) levels, which are known to stimulate aldosterone production *in vitro* (Xing et al., 2012), suggesting that any beneficial effect of pioglitazone on aldosterone levels may occur through its improvement of elevated VLDL levels. In addition, pioglitazone can activate PPARα in addition to PPARγ (Sakamoto et al., 2000), and in fact, this lack of PPARγ specificity appears to improve its safety profile relative to the more selective PPARγ agonists such as rosiglitazone (Winkelmayer et al., 2008). Therefore, the physiological role of PPARγ 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 (Wang and Rainey, 2012; Wang et al., 2012). 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 (Parmar et al., 2008). In this study, we investigated the regulation of human genes involved in aldosterone biosynthesis using pioglitazone and AngII.

2. Materials and methods

2.1. 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-eIF2α (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).

2.2. Cell culture and treatment

The human adrenocortical carcinoma HAC15 cell line (Parmar et al., 2008), which is a clone of the H295R cell line (Wang and Rainey, 2012; Wang et al., 2012), 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 μM pioglitazone in the presence and absence of 10 nM AngII in fresh low-serum experimental medium. For the dose response experiments, the supernatants were collected and cells were harvested after treatment for 24 h with 300 nM, 1 μM, 3 μM and 10 μM 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 μM pioglitazone for 4, 8, 12, 16, 24 or 36 h as indicated. For experiments examining StAR expression, cells were treated in two different ways: in one set of experiments cells were treated for 1 h with or without 10 nM AngII in the presence or absence of 10 μM pioglitazone; in the other, the cells were pretreated for 24 h with DMSO or 10 μM pioglitazone, followed by treatment with or without 10 nM AngII for 1 h. For some experiments, cells were treated with 10 μM pioglitazone and 10 μM GW9662 or GW6471 or 500 μM TUDCA for 24 h.

2.3. Measurement of steroid hormone production

HAC15 cells were incubated for 16–20 h 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 (Xing et al., 2011). 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.

2.4. 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 μg 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 20 s, 95 °C 1 s, 65 °C for 20 s for 40 cycles.

Relative mRNA expression values were determined by the $2^{-\Delta\Delta Ct}$ method using human cyclophilin (PPIA) as the normalization control. The cycle-to-threshold (Ct) values were calculated for statistical analysis. Correction for loading control, ΔCt, is calculated as $Ct_{target}-Ct_{reference}$, and reference is the Ct value for PPIA; ΔΔCt is expressed as $\Delta Ct_{treated}-\Delta Ct_{control.}$ Fold change in mRNA expression of each sample by treatment is then calculated by the formula $2^{-\Delta\Delta Ct}$ (Livak and Schmittgen, 2001). The probe sets for human CYP11B2 (Hs01597732_m1), CYP11B1 (Hs01596404_m1),