Thiazolidinediones (TZDs) such as pioglitazone and rosiglitazone are widely used as insulin sensitizers in the treatment of type 2 diabetes. In diabetic women with polycystic ovary syndrome, treatment with pioglitazone or rosiglitazone improves insulin resistance and hyperandrogenism, but the mechanism by which TZDs down-regulate androgen production is unknown. Androgens are synthesized in the human gonads as well as the adrenals. We studied the regulation of androgen production by analyzing the effect of pioglitazone and rosiglitazone on steroidogenesis in human adrenal NCI-H295R cells, which are derived from a tumor of the adrenal cortex. Both TZDs changed the steroid profile of the NCI-H295R cells and inhibited the activities of P450c17 and 3βHSDII, key enzymes of androgen biosynthesis. Pioglitazone but not rosiglitazone inhibited the expression of the CYP17 and HSD3B2 genes. Likewise, pioglitazone repressed basal and 8-bromo-cAMP-stimulated activities of CYP17 and HSD3B2 promoter reporters in NCI-H295R cells. However, pioglitazone did not change the activity of a cAMP-responsive luciferase reporter, indicating that it does not influence cAMP/protein kinase A/cAMP response element-binding protein pathway signaling. Although peroxisome proliferator-activated receptor γ (PPARγ) is the nuclear receptor for TZDs, suppression of PPARγ by small interfering RNA technique did not alter the inhibitory effect of pioglitazone on CYP17 and HSD3B2 expression, suggesting that the action of pioglitazone is independent of PPARγ. On the other hand, treatment of NCI-H295R cells with mitogen-activated protein kinase kinase (MEK)/extracellular signal-regulated kinase (ERK) inhibitor 2-[2-amino-3-methoxyphenyl]-4H-1-benzopyran-4-one (PD98059) enhanced promoter activity and expression of CYP17. This effect was reversed by pioglitazone treatment, indicating that the MEK/ERK signaling pathway plays a role in regulating androgen biosynthesis by pioglitazone.
ing affinity for PPARγ (Willson et al., 1996), there is growing evidence that the biological effects of TZDs expand beyond insulin-sensitizing and may not solely be receptor-mediated (Palakurthi et al., 2001; Feinstein et al., 2005). Apart from their metabolic actions PPARγ agonists exhibit antineoplastic effects on several tumor cell lines either through induction of apoptotic cell death or through production of reactive oxygen species. Thus TZD’s means of action remain open, and further details on the possible mechanisms of action need to be elucidated.

Pioglitazone and rosiglitazone are the TZDs that are currently in clinical use for the treatment of type 2 diabetes mellitus (T2DM). Several clinical studies have demonstrated that both pioglitazone and rosiglitazone not only are able to improve insulin sensitivity in patients with T2DM but may also decrease plasma androgen concentrations (DHEA, androstenedione) in women with polycystic ovary syndrome (PCOS) (Brettenthaler et al., 2004; Ortega-Gonzalez et al., 2005; Sepilian and Nagamani, 2005). Controversy exists as to whether the antian drogenic action of TZDs is mediated indirectly through their insulin-sensitizing effect, and it is unknown whether it depends on PPARγ.

PCOS is a common disorder of androgen excess. The pathophysiological mechanism(s) underlying PCOS are unknown, as is the exact physiological regulation of androgen biosynthesis. Elevated circulating androgen concentrations in women with PCOS may originate from the ovaries and/or the adrenal cortex (Lachelin et al., 1982; Barnes et al., 1989; Ehrmann et al., 1992). Enhanced androgen biosynthesis in PCOS is caused by an increased expression of the steroidogenic enzymes P450c17 (17α-hydroxylase, 17,20-lyase) and 3βHSDII, which are essential for the production of androgens (Nelson et al., 1999, 2001).

In this study, we investigated the regulation of human androgen biosynthesis using pioglitazone and rosiglitazone as chemical tools. Whereas previous in vitro studies demonstrated that TZDs may directly inhibit the enzymatic activities (3βHSDII and P450c17) (Arlt et al., 2001), we studied the effects of TZDs on cell signaling and gene expression in human adrenocortical NCI-H295R cells. NCI-H295R cells were chosen because this represents a well-established model to study the steroidogenesis of the human adrenal cortex (Gazdar et al., 1990; Staels et al., 1993). NCI-H295R cells express all genes that encode the steroidogenic enzymes present in all three layers of the adult adrenal cortex, including 3βHSDII and P450c17, key enzymes of androgen biosynthesis.

**Materials and Methods**

**Materials.** Pioglitazone was provided by Takeda Pharma AG (Lachen, Switzerland) and rosiglitazone by GlaxoSmithKline (Münchenbuchsee, Switzerland). PD98059 and 8-bromo-cAMP (8Br-cAMP) were purchased from Sigma (Buchs, Switzerland). The antibodies against phospho-MEK1 and phospho-ERK1/2 were purchased from Cell Signaling Technology (Danvers, MA), ERK1/2 from Santa Cruz Biotechnology Inc. (Santa Cruz, CA), PPARγ from Upstate (Lake Placid NY), and β-actin from Sigma. Goat anti-rabbit horseradish peroxidase-conjugated antibody was obtained from Santa Cruz Biotechnology. Radioactive-labeled [7(N)-3H]pregnenolone (14 Ci/mmol) and [1,2,6,7(N)-3H]DHEA (63 Ci/mmol) were procured from PerkinElmer (Boston MA), and [1,2,6,7(N)-3H]17α-hydroxypregnenolone (50 Ci/mmol) was acquired from American Radiolabeled Chemicals (St. Louis, MO). Trilostane was extracted in absolute ethanol from tablets commercially available as Modrenal (Biovision, New York, NY).

**Cell Culture and Treatment.** Human adrenal NCI-H295R cells were ordered from American Type Culture Collection (Manassas, VA). Cells were cultured under standard conditions in Dulbecco’s modified Eagle’s Ham’s F-12 medium (Invitrogen, Basel, Switzerland) supplemented with 5% Nul serum, 0.1% selenium/insulin/transferrin (SIT) plus, penicillin, and streptomycin (all from Invitrogen). Pioglitazone and rosiglitazone were dissolved in dimethyl sulfoxide (DMSO) at stock concentrations of 10 mM; final concentrations used for treatment were in the range of 2.5 to 10 μM for both drugs. Reported mean effective serum concentrations for the treatment of T2DM patients are 1 μM for pioglitazone (Cox et al., 2000) and 2.5 μM for pioglitazone (Zhong and Williams, 1996). 8Br-cAMP was diluted in phosphate-buffered saline at a concentration of 100 mM; final concentration for treatment was 0.5 mM. MEK/ERK inhibitor PD98059 was dissolved in DMSO at a concentration of 20 mM, and the final concentration for treatment was 10 μM. For RNA and protein extraction experiments and for steroid labeling experiments, cells were grown in six-well plates. Twenty-four hours after subculturing, medium was replaced, and treatment was added in normal growth medium for 48 h unless indicated differently. Control cells were treated with 0.1% (v/v) DMSO.

**RNA IsoLation and Semiquantitative RT-PCR.** Total RNA from NCI-H295R cells was extracted using a Nucleospin RNA kit (Macherey Nagel, Oensingen, Switzerland). Total RNA from human adrenal tissues was extracted using the TRIzol method as described by the manufacturer (Invitrogen). RNA was reverse-transcribed to cDNA using the Improtm RNA Transcriptase kit (Promega, Madison, WI) with either 0.5 μg of oligo(dT) or random hexamers (Promega) per 1 μg of RNA at 42°C for 1 h. Aliquots of cDNA obtained from 100 ng of RNA were taken for PCR reactions in a final volume of 25 μl. For the PCR, GoTaq Polymerase (Promega) and specific primers were used (Table 1) (Fluck and Miller, 2004; Huang et al., 2005).

PCR conditions were as follows: 1 min at 94°C, 1 min at 59°C to 60°C, 1 min at 72°C, 22 cycles for CYP17 and GAPDH, 25 cycles for HSD3B2 and CYTB5, and 32 cycles for PPARγ/PPARγ2. Aliquots of PCR products were electrophoresed on a 1.5% agarose gel, visualized with ethidium bromide, scanned, and quantified using an Alpha Imager 3400 (Alpha Innotech, San Leandro, CA). Intensities of specific bands were compared with GAPDH as internal control and expressed as a percentage of the control (DMSO-treated cells).

**Real-Time PCR.** The mRNA levels of CYP17 and HSD3B2 were assessed by real-time PCR using an ABI 7000 Sequence Detection System (Applied Biosystems, Foster City, CA). In brief, PCR reactions were performed in 96-well plates (Agene, Epsom, UK) using cDNA prepared as described above (50 ng/20 μl). We used TaqMan Universal PCR Mix and 1 μl of specific primers and probes obtained as assay-on-demand gene expression products (Applied Biosystems). Relative expression values were determined by the 2-△△Ct method using 18S rRNA as an internal control. Amplification curves and the mean Ct (threshold) values were calculated using the ABI Prism Sequence Detection System software (Applied Biosystems). Correction for internal control, ΔCt, is calculated as Ct_target − Ct_reference, and reference is the Ct value for 18S; ΔCt is expressed as ΔCt_corrected = ΔCt_target − ΔCt_control. Increase in RNA expression of each sample by treatment is then calculated by the formula 2-△△Ct (Livak and Schmittgen, 2001). Results are expressed as a percentage of DMSO control.

**Labeling of Steroidogenesis.** NCI-H295R cells were cultivated in normal growth medium in six-well plates and treated for 48 h with TZDs. Steroid metabolism was labeled by adding [3H] pregnenolone, [3H]17α-hydroxypregnenolone, or [3H]DHEA (all 220,000 cpm/35-mm well) dissolved in ethanol [maximum 0.5% (v/v) concentration] for 90 min, unless indicated otherwise. In some experiments, cells were pretreated with 1 μM trilostane for 2.5 h before adding labeled steroid. Steroids were extracted from the growth medium as...
described previously (Arlt et al., 2001), separated on thin-layer chromatography (TLC) plates (Whatman, Clifton, NJ) using chloroform/ethyl acetate (3:1) as solvent system, and then exposed and visualized on a Storm PhosphorImager (GE Healthcare, Little Chalfont, Buckinghamshire, UK). Spots corresponding to specific steroids were densitometrically quantified using ImageQuant Software (GE Healthcare).Steroid conversion was assessed by calculating the percentage of total radioactivity incorporated in a specific steroid hot spot compared with total radioactivity added to the reaction. To compare activities of P450c17 and 3βHSD with and without pioglitazone treatment, the conversion ratio of substrate to product was calculated (Table 2). Results of probes after treatment were expressed as a percentage of mock-treated controls (DMSO).

Specific activities of P450c17 in the presence of triolostane were calculated as a percentage of total radioactivity incorporated in 17α-hydroxyprogrenolone and DHEA for 17α-hydroxylase activity and as a percentage of total radioactivity incorporated in DHEA for 17,20-lyase activity (Fig. 2).

Protein Analyses. Cell protein extractions and Western blot analysis of ERK1/2 and MEK phosphorylation and PPARγ expression were performed according to the protocols provided by the manufacturer (Cell Signaling Technology). In brief, cells were treated, washed with ice-cold phosphate-buffered saline, and collected in lysis buffer (200 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, and protease and phosphatase inhibitors). Lysates were passed through 25-gauge needles, centrifuged for 15 min at 12,000g at 4°C, from which supernatants were then collected. Protein concentrations of cell extracts were measured by Bio-Rad protein assay (Bio-Rad Laboratories, München, Germany). Aliquots of protein extracts were resolved by 10% SDS-polyacrylamide gel electrophoresis and blotted on Immobilon P transfer membranes (Millipore, Billerica, MA) by semidy transfer method using the Trans-Blot SemiDry apparatus (Bio-Rad Laboratories, Hercules, CA). Blocking and staining of the membranes with antibodies were performed according to the manufacturer’s recommendations. Protein bands were visualized by enhanced chemiluminescence substrate reagent and exposed on ECL Plus films (GE Healthcare). Films were scanned by the Alpha Imager so as to quantify intensities of protein bands. β-Actin was used as a control for equal loading.

Plasmid Constructs and Transient Transfection of the Cells. Promoter activities of CYP17 and HSD3B2 were characterized by transient transfection of NCI-H295R cells with luciferase reporter gene constructs. Constructs of the long promoter (—3.7 kb) and the short basal promoter (—227 bp) of CYP17 in pMGL3 vector have been described earlier (Fluck and Miller, 2004). The HSD3B2 promoter construct was newly built by amplifying the promoter region (—1050/+55 bp) of the HSD3B2 gene using the following primers: sense, 5’-CGGCCCTATCAAGGCTCACTGTGATTTTTA-3’; and antisense, 5’-GATCTGAAGACCTCAGTACTGTTTTAAAGTCGACAGA-3’ (restriction sites are underlined). The fragment was cloned into polylinker of the pGL3 basic vector (Promega) using HindIII and XhoI restriction sites. The final construct was verified by direct

### Table 1
Sequences of primers used for semiquantitative RT-PCR and of oligonucleotides used for cloning of shPPARγ constructs

<table>
<thead>
<tr>
<th>CYP17-S (Fluck and Miller, 2004)</th>
<th>HSD3B2 (Huang et al., 2005)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Sense</strong></td>
<td><strong>Sense</strong></td>
</tr>
<tr>
<td>3βHSD</td>
<td>SULT2A1</td>
</tr>
<tr>
<td>Antisense</td>
<td>Antisense</td>
</tr>
<tr>
<td>PPARγ</td>
<td>PPARγ</td>
</tr>
<tr>
<td>GADPH</td>
<td>CYTBS (Fluck and Miller, 2004)</td>
</tr>
<tr>
<td><strong>Sense</strong></td>
<td><strong>Sense</strong></td>
</tr>
<tr>
<td>Antisense</td>
<td>Antisense</td>
</tr>
</tbody>
</table>

### Table 2
Effect of pioglitazone on the activities of P450c17 and 3βHSDII

Enzyme activities were assessed by calculating the substrate to product conversion ratios from data shown in Fig. 1. Data are expressed as the percentage of control and represent the mean of three independent experiments ± S.D.

<table>
<thead>
<tr>
<th>Enzyme Activities</th>
<th>Basal</th>
<th>cAMP-Stimulated</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DMSO 100 µM PIO</td>
<td>DMSO 10 µM PIO</td>
</tr>
<tr>
<td>17α-Hydroxylase</td>
<td></td>
<td></td>
</tr>
<tr>
<td>17α-Hydroxylase</td>
<td>100%</td>
<td>100%</td>
</tr>
<tr>
<td>17α-Hydroxylase</td>
<td>109 ± 15</td>
<td>100%</td>
</tr>
<tr>
<td>17α-Hydroxylase</td>
<td>63 ± 7</td>
<td>100%</td>
</tr>
<tr>
<td>17α-Hydroxylase</td>
<td>81 ± 5</td>
<td>100%</td>
</tr>
<tr>
<td>17α-Hydroxylase</td>
<td>64 ± 5</td>
<td>100%</td>
</tr>
<tr>
<td>17α-Hydroxylase</td>
<td>76 ± 12</td>
<td>100%</td>
</tr>
</tbody>
</table>

A. androstenedione; Preg, pregnenolone; 17α-Hydroxyprogesterone; 17α-Hydroxyprogrenolone.
sequencing (Microsynth, Balgach, Switzerland). Reporter construct pCMVluc which is a luciferase expression vector under the control of 16 cAMP-responsive elements has been described elsewhere (Fluck et al., 2002). The mammalian expression vector containing the human PPARγ1 cDNA was a generous gift of Dr. Chatterjee (Cambridge, UK).

For the transient transfection, NCI-H295R cells were split into 24-well plates at a density of approximately 200,000 cells/well and incubated in transfection medium (Dulbecco’s modified Eagle’s Ham’s F-12 medium containing 10% Nul serum, supplemented with 0.1% SIT). The next day, transfection was carried out for 6 h at 37°C using 1.6 μl of Lipofectamine 2000 (Invitrogen, Carlsbad, CA), 10 ng of Renilla reniformis luciferase in a pCMV vector (pRL-CMV, an internal control normalizing for cell number and transfection efficiency), and 0.5 μg of specific firefly luciferase reporter plasmids. After transfection, the medium was replaced by growth medium, and cells were allowed to recover for 2 to 3 h before adding TZDs for 48 h. In some experiments, cells were stimulated with 0.5 mM 8Br-cAMP. Finally, cells were lysed and assayed for dual luciferase activities as described by the manufacturer (Promega).

Silencing of PPARγ Expression. Oligonucleotides for silencing of PPARγ were designed with Ambion design software (Ambion, Austin, TX). Sequences of oligonucleotides (oligo1 and 2) are given in Table 1. Oligonucleotides were annealed and cloned into BglII and HindIII cloning sites of pSUPER basic vector (Oligoengine, Seattle, WA). Correct cloning of constructs was confirmed by direct sequencing (Microsynth). NCI-H295R cells were transiently transfected with either an empty vector or shPPARγ constructs in suspension. In brief, cells were treated with transfection mixture containing plasmid (4 μg/well) and Lipofectamine (9 μl/well) and then seeded onto six-well plates (1 × 10^6 cells/well). Cells were allowed to transfect and adhere for 6 h, then they were washed, and normal growth medium was added. Forty-eight hours after transfection, either DMSO or 10 μM pioglitazone was added to the growth medium for the next 36 h before the cells were collected for RNA extraction. Efficiency of transfection (60–70%) was monitored microscopically using enhanced green fluorescent protein expression vector (Promega) as a control.

Statistics. All data represent the mean of at least three independent experiments. Experimental variation is given as SD. Statistical analysis was performed using the two-tailed Student’s t test (Excel; Microsoft Corp., Redmond, WA). Results

Pioglitazone and Rosiglitazone Inhibit Androgen Production in NCI-H295R Cells. The gonads and the human adrenal cortex synthesize androgens from cholesterol metabolites through the same steroidogenic pathway. Clinical studies have shown that TZDs such as pioglitazone lower the basal and the “adrenocorticotropic hormone (ACTH)-stimulated” plasma androgen concentrations in women with PCOS experiencing hyperandrogenism (Brettenthaler et al., 2004; Guido et al., 2004; Ortega-Gonzalez et al., 2005). In an in vitro model, TZD troglitazone has been shown to inhibit basal and luteinizing hormone (LH) and insulin-stimulated androgen production in human ovarian theca cells (Veldhuis et al., 2002).

We studied the effect of pioglitazone and rosiglitazone on steroidogenesis in human adrenal NCI-H295R cells. Cells were treated with 2.5 to 10 μM pioglitazone or rosiglitazone for 48 h, and steroidogenesis was assessed with [3H]pregnenolone labeling, which is the main precursor for all steroid synthesis. Overall, we found that both TZDs modified the steroidogenic profile of NCI-H295R cells, resulting in a decrease in androstenedione production and a decrease in the use of pregnenolone under both basal and 8Br-cAMP-stimulated conditions (Fig. 1). Calculations of substrate-to-product conversion ratios suggested a possible inhibition of the enzymatic activities of P450c17 and 3βHSDII, which was found to reach statistical significance for pioglitazone (Table 2).

Pioglitazone and Rosiglitazone Inhibit Activities of P450c17 and 3βHSDII in NCI-H295R Cells. Incubating NCI-H295R cells with [3H]pregnenolone will label the whole steroid pathway, because pregnenolone is the common precursor. Therefore, to measure the effect of TZDs on P450c17 activities in particular, we blocked 3βHSDII in NCI-H295R cells with 1 μM trilostane, a specific 3βHSD inhibitor. For the assessment of 17α-hydroxylase and 17,20-lyase activities, we treated NCI-H295R cells with trilostane and labeled steroidogenesis with [3H]pregnenolone before quantifying the conversion of pregnenolone to 17α-hydroxypregnenolone and DHEA (Fig. 2A). For the assessment of 17,20-lyase activity alone, steroidogenesis was labeled with [3H]17α-hydroxypregnenolone, and conversion to DHEA was quantified (Fig. 2B). Treatment of NCI-H295R cells with either 10 μM pioglitazone or rosiglitazone for 48 h decreased both activities of P450c17 significantly (Fig. 2, A and B).

Likewise, to assess the effect of TZDs on 3βHSDII activity, we treated NCI-H295R cells with 10 μM pioglitazone or rosiglitazone and labeled steroidogenesis with [3H]DHEA, a specific substrate of 3βHSDII that will be converted to androstenedione. In our experiments both TZDs inhibited 3βHSDII activity (Fig. 2C).

Pioglitazone Inhibits Expression of Genes Involved in Androgen Production. Because TZDs inhibit the multistep metabolism of pregnenolone through the steroidogenic pathway, they may inhibit steroidogenic enzymes either by repressing the expression of these enzymes or by direct inhibition of enzymatic activities. In fact, pioglitazone and other TZDs have been shown to inhibit enzymatic activities of P450c17 and 3βHSDII directly in vitro (Arti et al., 2001), but this inhibition was observed only at supraphysiological concentrations of these drugs. Thus, we studied the effect of pioglitazone on steroidogenesis in NCI-H295R cells by assessing the expression of the involved genes at the mRNA level. Analysis of RNA expression by semiquantitative RT-PCR revealed that treatment with 2.5 to 10 μM pioglitazone down-regulated basal expression of CYP17 and HSD3B2 (Fig. 3A). Stimulation of NCI-H295R cells by 8Br-cAMP activated the expression of both CYP17 and HSD3B2 genes, but the inhibitory effect of pioglitazone persisted (Fig. 3A). In contrast, pioglitazone did not influence the expression of genes for microsomal cytochrome b5α, a protein that supports 17,20-lyase activity of P450c17, and for sulfotransferase SULT2A1, the enzyme responsible for conversion of DHEA to DHEA-S (data not shown). To better quantify the effect of pioglitazone on gene expression, we performed quantitative real-time PCR. We found that pioglitazone inhibited CYP17 and HSD3B2 expression at concentrations greater than 2.5 μM, down-regulating CYP17 to 40% and HSD3B2 to ~50% at 10 μM concentration. Stimulation with 0.5 nM 8-Br-cAMP for 24 h induced the expression of both genes at the RNA level by 3- to 4.5-fold, but pioglitazone was also able to inhibit the expression of CYP17 (~40%) and HSD3B2 (~70%) in the stimulated state (Fig. 3B).
Pioglitazone Represses Promoter Activities of CYP17 and HSD3B2. We transiently transfected NCI-H295R cells with promoter-luciferase reporter constructs and treated them with 10 μM pioglitazone for 48 h, with and without 8Br-cAMP stimulation, to assess the influence of pioglitazone on transcriptional regulation of CYP17 and HSD3B2. Pioglitazone was found to inhibit activities of both long -3.7 kb and basal -227 bp CYP17 promoter reporter by 27 to 35% (Fig. 4). Stimulation with 0.5 mM 8Br-cAMP increased activities of the CYP17 constructs approximately 3-fold, but pioglitazone treatment again led to a repression of these activities by 44 to 55% (Fig. 4). In contrast, pioglitazone treatment did not change basal activity of the -1.05-kb reporter construct of the HSD3B2 promoter, although the expression of its mRNA was decreased more than CYP17. Because artificial “promoter-reporter” assays allow the use of chosen parts of promoters only, essential elements, which are modulated by pioglitazone, may be missed. Recently, a specific intronic sequence of the HSD3B gene has been reported to be important for basic promoter activity of both HSD3B2 and HSD3B1 genes (Foti and Reichardt, 2004), corroborating our hypothesis. Alternatively, pioglitazone might affect the stability of HSD3B2 mRNA. However, after stimulating NCI-H295R cells with 0.5 mM 8Br-cAMP, the activity of the transiently transfected HSD3B2 promoter reporter increased by 13-fold, and pioglitazone treatment inhibited this activity by approximately 50% (Fig. 4). Thus, pioglitazone showed an inhibitory effect on promoter activities of both CYP17 and HSD3B2 genes.

![Fig. 1. Effect of pioglitazone and rosiglitazone on steroid production in NCI-H295R cells. Cells were treated with 2.5 to 10 μM pioglitazone (PIO) or rosiglitazone (ROS) for 48 h and optionally stimulated with 0.5 mM 8Br-cAMP. Steroidogenesis was labeled using [3H]pregnenolone (220,000 cpm/35-mm well) as a substrate for 90 min. Steroids were extracted from media and resolved on TLC plates. A, representative TLC showing effect of pioglitazone (left) and rosiglitazone (right) treatment on steroidogenic profile of NCI-H295R cells. B and C, quantification of basal and cAMP stimulated steroidogenesis for NCI-H295R cells treated with 5 to 10 μM pioglitazone (B) or 5 to 10 μM rosiglitazone (C). Results are expressed as a percentage of total radioactivity incorporated in specific steroid product. Data represent the mean of three to four independent experiments, error bars are S.D. A, androstenedione; Preg, pregnenolone; 17OH Prog, 17α-hydroxyprogesterone; 17OH Preg, 17α-hydroxypregnenolone; 11 DOC, 11-deoxy cortisol.](image-url)
Rosiglitazone Does Not Change Gene Expression of CYP17 and HSD3B2. Clinical studies have demonstrated that treatment with pioglitazone and rosiglitazone lowers elevated plasma androgen concentrations in women with PCOS (Brettenthaler et al., 2004; Ortega-Gonzalez et al., 2005; Sepilian and Nagamani, 2005). Analysis of the activities of P450c17 and 3βHSDII in NCI-H295R cells treated with rosiglitazone and pioglitazone revealed a similar pattern, although the effect of rosiglitazone seemed to be weaker in quantity (Figs. 1C and 2). Thus, we tested whether rosiglitazone would also change the expression of CYP17 and HSD3B2 genes similar to pioglitazone. Semiquantitative and quantitative real-time PCR experiments were performed on reverse-transcribed RNA extracted from NCI-H295R cells treated with different concentrations of rosiglitazone. In contrast to pioglitazone, rosiglitazone did not change the expression of the CYP17 and HSD3B2 genes significantly, even after stimulation with 0.5 mM 8Br-cAMP (Fig. 5, A and B). Likewise, rosiglitazone did not change the activities of CYP17 and HSD3B2 promoter reporter constructs transfected into NCI-H295R cells (Fig. 5C). These data suggest that unlike pioglitazone, rosiglitazone does not affect androgen production in NCI-H295R cells by modulating expression of genes essential for the biosynthesis of androgens. Therefore, we focused further studies on the regulation of the CYP17 and HSD3B2 genes by pioglitazone.

Pioglitazone Does Not Signal through the cAMP/PKA/CREBP. Glucocorticoid and androgen production in the human adrenal cortex may be stimulated by the ACTH/cAMP/PKA pathway. Because pioglitazone lowers the ACTH-stimulated androgen production in women with PCOS (Guido et al., 2004), and because we found that pioglitazone treatment repressed 8Br-cAMP-stimulated expression of CYP17 and HSD3B2 in NCI-H295R cells (Figs. 3 and 4), we tested the hypothesis that pioglitazone might interfere with the classic cAMP/PKA/CREBP pathway. We transfected NCI-H295R cells with the luciferase reporter construct pCRE-luc, which is driven by cAMP-dependent proteins binding to 16 CREs in the promoter (Fluck et al., 2002). Stimulation with 8Br-cAMP activated this luciferase reporter 2.5-fold, and pioglitazone treatment did not alter this stimulation (Fig. 6). Because pioglitazone did not change luciferase activity of pCRE-luc, we can also exclude that pioglitazone may directly inhibit the enzymatic activity of luciferase. These findings suggest that pioglitazone does not inhibit androgen biosynthesis in NCI-H295R cells by interfering with the regulation of CYP17 and HSD3B2 genes through cAMP/PKA/CREBP signaling.

Pioglitazone Counteracts Regulation of CYP17 Gene Expression by ERK. Previous studies have demonstrated
that the expression of the CYP17 gene in NCI-H295R cells is regulated through the MAPK pathway (Sewer and Waterman, 2003a). Moreover, theca cells isolated from ovaries of women with PCOS were found to have decreased activity of MEK and ERK1/2 kinases, a finding which is in line with enhanced expression of CYP17 mRNA and increased production of androstenedione observed in these cells (Nelson-Degrave et al., 2005). To study the possible regulation of CYP17 and HSD3B2 by the MAPK pathway in NCI-H295R cells, we compared expression and promoter activities of these genes under the influence of the MEK inhibitor PD98059. Quantitative real-time PCR analysis of gene expression revealed that treatment with 10 μM PD98059 enhanced CYP17 expression 3-fold and HSD3B2 expression 2.5- to 4-fold (Fig. 7A). However, when NCI-H295R cells were treated with pioglitazone, ERK inhibition did not increase CYP17 expression. The observed effect of pioglitazone treatment and ERK inhibition on HSD3B2 expression was similar, but statistical analysis did not reach significance. Likewise, ERK inhibition by PD98059 enhanced the activity of the CYP17 promoter reporter in NCI-H295R cells significantly, and pioglitazone treatment repressed this activation. In contrast, PD98059 treatment alone or in combination with pioglitazone did not change the activity of the HSD3B2 promoter construct significantly when transfected into NCI-H295R cells (Fig. 7B).

To analyze the phosphorylation status of ERK1/2 in NCI-H295R cells, which directly correlates with ERK activity, we performed a Western blot (Fig. 7, C and D). Pioglitazone slightly increased while 10 μM PD98059 partially decreased the phosphorylation of ERK (P-ERK) in NCI-H295R cells. It is interesting that, when treating NCI-H295R cells with both pioglitazone and the ERK inhibitor PD98059, P-ERK remained moderately up-regulated, similar to pioglitazone treatment alone. Phosphorylation of MEK (P-MEK), the kinase which is responsible for the phosphorylation and thus activation of ERK, was not influenced by pioglitazone or PD98059 (Fig. 7C). Thus, pioglitazone might interfere with MAPK signaling at this level or downstream of the MEK kinase. Stimulation of NCI-H295R cells with 8Br-cAMP markedly induced the amount of P-ERK and slightly of P-MEK, but pioglitazone treatment did not alter this activa-

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tion, thus confirming our previous suggestion that pioglitazone does not affect cAMP/PKA signaling (Fig. 7C).

To further assess the influence of pioglitazone on ERK-regulated steroidogenesis, we analyzed the activities of P450c17 and 3βHSDII in NCI-H295R cells under PD98059 treatment with or without pioglitazone (Fig. 7, E and F). Partial inhibition of ERK resulted in an increase in 17,20-lyase activity of P450c17 and consequently more DHEA production when measured in the presence of 1 μM trilostane. Consistent with previous experiments, pioglitazone inhibited the increase in P450c17 activity (Fig. 7, E, top, and F). In contrast, using [3H]DHEA as a specific substrate, we found that 3βHSDII activity was not significantly reduced by PD98059, but pioglitazone still decreased 3βHSDII activity in its presence (Fig. 7, E and F).

The Effect of Pioglitazone on CYP17 and HSD3B2 Expression Seems to be PPARγ-Independent. TZDs like pioglitazone are believed to be ligands for PPARγ, which belong to the large group of nuclear receptors. To evaluate whether the effect of pioglitazone on androgen production might be PPARγ-mediated, we first analyzed human adrenal NCI-H295R cells and adult human adrenal tissues for PPARγ expression. PPARγ has two splice variants, PPARγ1, which is known to be expressed in many tissues, and PPARγ2, the specific variant for white adipose tissue (Kota et al., 2005). Analysis of reverse-transcribed RNA by PCR revealed that both human adrenals and human adrenal NCI-H295R cells express PPARγ1 but not PPARγ2 (Fig. 8A).

To suppress PPARγ expression in NCI-H295R cells, we built two vectors for targeted PPARγ silencing (Table 2). Transfection of NCI-H295R cells with these constructs caused a significant decrease in PPARγ expression at RNA and protein level regardless of whether cells were treated with 10 μM pioglitazone or with DMSO as a vehicle (Fig. 8, B and C). Pioglitazone treatment slightly increased the expression of PPARγ (Fig. 8B). Despite effective silencing of PPARγ in NCI-H295R cells, pioglitazone inhibited both CYP17 and HSD3B2 gene expression to the same extent as in NCI-H295R control cells (Fig. 8D), and silencing of PPARγ did not influence basal levels of CYP17 and HSD3B2 expression (data not shown). Likewise, transfection of NCI-H295R cells with a pCDNA3-PPARγ vector for overexpressing PPARγ did not change the effect of pioglitazone on CYP17 and HSD3B2 gene expression (data not shown). These data indicate that PPARγ is not required for mediating the inhibitory action of pioglitazone on CYP17 and HSD3B2 gene expression.

Discussion

In this study, we analyzed the mechanisms/pathways by which pioglitazone and rosiglitazone regulate the biosynthesis of androgen production in the human adrenals. We demonstrated that both TZDs are able to lower androgen production in steroidogenic human adrenal NCI-H295R cells. Pioglitazone but not rosiglitazone repressed transcription and expression of CYP17 and HSD3B2 genes, which encode the essential enzymes for androgen biosynthesis. It is interesting that for NCI-H295R cells, an inhibitory effect of pioglitazone on both CYP17 promoter activation and gene expression was found not only in the basal state but also when stimulated with cAMP. Likewise, pioglitazone inhibited HSD3B2 gene expression under both basal and cAMP-stimulated conditions, but it repressed activity of HSD3B2 promoter construct only when stimulated with cAMP, indicating that the promoter construct did not contain the essential regulatory elements. These results suggest that pioglitazone may down-regulate CYP17 and HSD3B2 transcription and gene expression independently of the cAMP/PKA signaling cascade. In support of this, we also found that pioglitazone did not change the activity of a CAMP-responsive reporter construct when transfected into NCI-H295R cells. However, because inhibition of CYP17 and HSD3B2 by pioglitazone was even stronger when NCI-H295R cells were stimulated with cAMP, pioglitazone may act through a signaling cascade that links to the cAMP/PKA pathway.

The cAMP/PKA pathway is the major signaling pathway regulating steroidogenesis (Stocco et al., 2005). cAMP is the main secondary messenger that is generated after stimulation of the gonadotropin receptors in the gonads and the ACTH receptor in the adrenal cortex (Sewer and Waterman, 2003a). Typically, cAMP activates PKA, which then induces gene transcription by phosphorylating and thus activating CREBP. Activities of most steroidogenic enzymes, including P450c17 and 3βHSDII, may be stimulated by cAMP in the so-called “long-term” response, a process which seems to require synthesis of new proteins because it was shown to be sensitive to inhibition of translation by cycloheximide (Waterman and Bischof, 1996). However, conflicting with classic cAMP/PKA signaling, promoters of genes for most steroidogenic enzymes do not contain typical CREs. In murine Leydig cells, LH-stimulated expression of CYP17 involves cAMP/PKA but is independent of CREB (Laurich et al., 2002). In addition, several reports have established the role of a possible cross-talk between cAMP/PKA and other signaling pathways such as MEK1/ERK or phosphatidylinositol 3-kinase/protein kinase B (Richards, 2001). In mouse Leydig cells, LH and 8Br-cAMP were shown to stimulate phosphorylation of ERK by PKA-dependent activation of Ras (Hirakawa and Ascoli, 2003). In human adrenal NCI-H295R cells, cAMP-mediated transcription of the CYP17 gene was
demonstrated to be dependent on the activity of dual specificity phosphatase, which was later identified as MAPK phosphatase-1 (Sewer and Waterman, 2002). MAPK phosphatase-1 may directly dephosphorylate kinase ERK1/2 and transcription factor SF-1, which is essential for the expression of almost all steroidogenic genes (Sewer and Waterman, 2003b).

Compared with healthy human theca cells, theca cells in women with PCOS have a significantly altered MEK/ERK phosphorylation status, leading to higher expression of the CYP17 gene and enhanced androstenedione production (Nelson-Degrave et al., 2005). Consistent with this, treatment of normal human theca cells with the MEK inhibitor PD98059 leads to higher abundance of CYP17 mRNA and higher activity of CYP17 promoter constructs in luciferase assays. Likewise, we can demonstrate that partial inhibition of the ERK pathway increases the expression of the CYP17 and HSD3B2 genes and increases the activity of the CYP17 promoter in human adrenal NCI-H295R cells. ERK inhibition seems to predominantly enhance the 17,20-lyase activity. In addition, we show for the first time that these effects may be antagonized by pioglitazone. Moreover, pioglitazone increases phosphorylation of ERK1 in the presence of the inhibitor PD98059 without affecting phosphorylation of MEK, indicating that it may modulate ERK downstream of MEK. Therefore, we suggest that pioglitazone inhibits androgen production, at least in part, through a modulatory effect on ERK phosphorylation.

Fig. 7. Pioglitazone antagonizes the effect of the MEK/ERK inhibitor PD98059 on CYP17 expression and promoter activity in NCI-H295R cells. A, NCI-H295R cells were treated with 10 μM PIO and 10 μM PD98059 for 48 h. Total RNA was isolated, and expression of CYP17 and HSD3B2 analyzed by real-time PCR. B, promoter activities of basal CYP17 promoter and 1.05-kb HSD3B2 promoter were assessed after transient transfection of luciferase reporter constructs into NCI-H295R cells followed by treatment with 10 μM PIO and/or 10 μM PD98059 for 48 h. C, to analyze phosphorylation status of MEK and ERK, NCI-H295R cells were treated with 10 μM PIO and/or 10 μM PD98059 for 48 h (optionally stimulated with 0.5 mM 8Br-cAMP for 24 h). Protein extracts were prepared and analyzed by Western blot. D, quantification of P-ERK from three independent experiments as described in C. E and F, effect of 10 μM PD98059 alone or in combination with 10 μM PIO on the activities of P450c17 and 3βHSD II in NCI-H295R cells. 17α-Hydroxylase and 17,20-lyase activities were assessed by labeling steroidogenesis with [3H]pregnenolone in the presence of 1 μM trilostane, an inhibitor of 3βHSD, for 90 min. Activity of 3βHSD II was assessed as the conversion of [3H]DHEA to androstenedione. Steroids were extracted and resolved on TLC. E, representative TLCs: P450c17 activities on the top, 3βHSD II on the bottom. F, quantification of three independent experiments as depicted in E. Enzymatic activities were quantified as the amount of total radioactivity incorporated in particular product and expressed as a percentage of DMSO control. *, p < 0.02; **, p < 0.05; , p < 0.02 compared with PD98059 treatment alone. A, androstenedione; Preg, progesterone; 17OH Preg, 17α-hydroxyprogrenolone.
Although both pioglitazone and rosiglitazone are TZDs and both lower plasma androgen levels in women suffering from PCOS, we find that only pioglitazone inhibits CYP17 and HSD3B2 gene transcription and expression. Thus, in NCI-H295R cells, molecular mechanisms of the androgen-lowering action of pioglitazone and rosiglitazone must be at least partially different. Differing effect of pioglitazone compared with rosiglitazone has been reported in terms of basal and follicle-stimulating hormone-stimulated estradiol production in human ovarian cells (Seto-Young et al., 2005). In that study, pioglitazone seemed to be significantly more potent, but the effect on progesterone production and insulin-induced inhibition of insulin-like growth factor binding protein 1 was similar for both TZDs. In contrast, a former in vitro study showed that both pioglitazone and rosiglitazone repress enzymatic activities of recombinant P450c17 and 3β-HSDII directly (Arlt et al., 2001). Direct enzymatic inhibition is strongest for troglitazone at 10 to 20 μM concentrations followed by rosiglitazone (50–100 μM) and pioglitazone at highly supratherapeutic concentrations (>100 μM). Thus, the effect of pioglitazone on androgen production seems to be predominantly at the level of gene regulation, and rosiglitazone may directly modulate the activities of these enzymes. However, from our studies, we cannot exclude that rosiglitazone may also act through other signaling cascades regulating targeted enzymes at the posttranslational level.

TZDs are ligands for PPARγ. Therefore, it seems compelling to hypothesize that their influence on androgen production might be mediated through the nuclear receptor PPARγ. NCI-H295R cells and human adrenal tissues express PPARγ splice variant 1 which is highly expressed in liver, adipose tissue, and macrophages. It is interesting that silencing or overexpressing PPARγ in NCI-H295R cells did not change the inhibitory effect of pioglitazone on CYP17 and HSD3B2 expression. Thus, we conclude that pioglitazone influences steroidogenic gene expression independently of its nuclear receptor. For inhibiting gene expression, we used pioglitazone in slightly higher doses (5–10 μM) than is used in the

Fig. 8. Effect of pioglitazone on gene expression of CYP17 and HSD3B2 is PPARγ independent. A, analysis of expression of PPARγ and its splice variant PPARγ2 in NCI-H295R cells and in adult human adrenal tissues. PCR was performed on 150 ng of reverse-transcribed total RNA, aliquots of reaction were loaded on a 1.5% agarose gel and visualized by staining with ethidium bromide. B, silencing of PPARγ expression in NCI-H295R cells using two different shPPARγ oligonucleotides cloned into pSUPER expression vector. Cells were transfected in suspension with given constructs using Lipofectamine 2000 (Invitrogen), cultivated for 72 h and treated with 10 μM pioglitazone for an additional 36 h. Total RNA was isolated, and expression of PPARγ was analyzed by RT-PCR. GAPDH amplification was used as internal control. C, Western blot analysis of silencing of PPARγ. Cells were transfected as described for 48 h, and proteins were extracted and analyzed by SDS-polyacrylamide gel electrophoresis and Western blotting as described under Materials and Methods. Two different amounts of shPPARγ oligonucleotide 1 were used; low, 0.4 μg of plasmid/well; and normal, 4 μg of plasmid/well. D, inhibition of CYP17 and HSD3B2 gene expression by 10 μM pioglitazone in cells transfected by either empty vector or shPPARγ oligonucleotides. Results are shown as percentage of pioglitazone versus DMSO control. Analysis was performed by real-time PCR on the reverse-transcribed RNA isolated for PPARγ expression analysis (Fig. 8B). All data represent the mean of three independent experiments. Error bars are S.D.; *, p < 0.02.
clinical setting of patients with T2DM (1–5 μM) (Zhong and Williams, 1996). But direct comparison of effective drug concentrations in vivo and in vitro may be inappropriate for many reasons such as binding of the drug to proteins or accumulation in tissues. Effective concentrations used in vivo and in vitro greatly exceed reported IC_{50} affinity constants for TZD binding to PPARγ, which were calculated to be 0.55 μM for pioglitazone and 0.075 μM for rosiglitazone (Willson et al., 1996). Thus, the fact that the antiangiogenic effect of pioglitazone is not PPARγ-mediated may also explain why studies of the binding affinity of TZDs and PPARγ do not necessarily correlate with their biological activity. Others have reported PPARγ-independent effects of TZDs (Feinstein et al., 2005). Perhaps the most interesting findings in line with our results are the observations that PPARγ ligands such as cigitazone, rosiglitazone, and pioglitazone increase ERK activity in vascular smooth muscle cells (Takeda et al., 2001) and transactivate the epidermal growth factor receptor in rat liver cells independent of PPARγ (Gardner et al., 2003). In summary, pioglitazone inhibits androgen production and the expression of the CYP17 and HSD3B2 genes in NCI-H295R cells, at least in part, through modulation of ERK signaling, and this effect is PPARγ-independent. We cannot exclude the possibility that pioglitazone uses additional pathways for modulating the production of androgens. There is growing evidence that TZDs have further mechanisms of actions including direct interactions with mitochondrial metabolism or with the activities of essential transcription factors, which are mostly regulated by (de-)phosphorylation. TZDs are widely used for the treatment of common diseases such as T2DM and PCOS. The spectrum of the clinical use of TZDs might even broaden in the near future given their possible anti-inflammatory potential, action as tumor suppressors, or anti-atherogenic effect (Michalik et al., 2004; Feinstein et al., 2005). Thus, our findings underline the importance of characterizing biological effects of each single TZD specifically because their mode of action seems to be compound-specific rather than a characteristic of all TZDs.

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