Regulation of steroid hormone biosynthesis enzymes and organic anion transporters by forskolin and DHEA-S treatment in adrenocortical cells

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The precursor of all steroid hormones is cholesterol, which is a 27-carbon steroid nucleus. The cells of the steroidogenic tissues can de novo synthesize cholesterol from acetate, mobilize the intracellular cholesterol ester pools, or import lipoprotein from the plasma. Cholesterol is stored as cholesterol acetate in neutral lipid droplets, which serve as a pool of readily available cholesterol for corticosteroid biosynthesis (20). About 80% of cholesterol is usually provided by circulating plasma lipoproteins as low-density lipoproteins (LDL) (12). Cholesterol is converted to steroid hormone intermediates and mature hormones by cytochrome P-450 enzymes. Cortisol synthesis begins in the mitochondria, continues in the endoplasmic reticulum, and is completed in the mitochondria. Therefore, shuttling of steroid hormone precursors between the mitochondria and cytoplasmic compartments is important in the multiple steps of hormone synthesis.

The rate-limiting steps in steroidogenesis are cholesterol transport across the outer and inner mitochondrial membranes and the CYP11A1 (20, 22-R-hydroxylase cholesterol side-chain cleavage) complex. The protein responsible for this transport, and as such regulating the acute production of steroids, has been identified and named steroidogenic acute regulatory protein (STAR) (21).

There have been almost 50 different steroids recognized as adrenal cortex products, which cover a wide range of physiological activities. In most species, including the human, the physiologically most important corticosteroids are aldosterone, cortisol, and dehydroepiandrosterone sulfate (DHEA-S). The adrenal cortex also produces estrogen, progesterone, and a wide range of precursors and metabolites of these steroids. In the medulla, norepinephrine and epinephrine are major secretory products that are derivatives of the amino acid tyrosine.

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Steroid and 11-deoxycorticosterone to the glucocorticoids corticosterone and cortisol, respectively.

There is growing evidence for the involvement of transporter proteins in the translocation of cortisol. Using the stop-flow peritubular capillary microperfusion method, Üllrich et al. (19) described cortisol transport across the basolateral membrane of the proximal tubule cell of the rat kidney. Recent studies showed an inhibition of cortisol release from primary cultures of bovine adrenocortical cells by probenecid and trans-stimulation of cortisol release by p-aminohippurate (PAH) and glutarate in the incubation medium (17). Further investigations on bovine adrenocortical cells demonstrated an uptake of radioactively labeled PAH, which was inhibited by probenecid and unlabeled PAH. The uptake of organic anions (e.g., PAH) into the cells, as well as the cortisol release from the cells, was stimulated by ACTH (18). Investigations applying RT-PCR on rat adrenals revealed the expression of organic anion transporter 1 (OAT1). In situ hybridizations and immunohistochemical analyses localized rat OAT1 to the zona fasciculata of the rat adrenal cortex, where cortisol synthesis and release take place. Importantly, OAT1 mRNA expression was strongly increased by treatment of rats with ACTH in vivo (2). Recently, we (1) reported a transporter protein-mediated translocation of cortisol in human (h)OAT3-expressing oocytes, and we also demonstrated probenecid-, cimetidine-, and glutarate-inhibitable estrone sulfate (ES) uptake by forskolin-treated human adrenocortical cells (NCI-H295R). ES uptake into NCI-H295R cells was trans-stimulated by preloading the cells with glutarate and cortisol, indicating the involvement of an organic anion transporter.

In this study, we investigated the influence of DHEA-S and ES on cortisol release by NCI-H295R cells compared with forskolin stimulation. This cell line was established in 1990 by Gaspar and coworkers from a patient with an adrenocortical carcinoma who showed a high secretion of mineralocorticoids, glucocorticoids, and androgens (11). Depending on the culture condition, these cells produce mainly C19 steroids and glucocorticoids. After treatment with forskolin, however, 70% of the released steroids are cortisol (14). We observed that, similarly to forskolin, pretreatment of NCI-H295R cells with DHEA-S increased cortisol release. Therefore, we investigated the impact of DHEA-S and forskolin on the expression of hOAT3, hOAT4, and key enzymes of glucocorticoid synthesis.

**MATERIALS AND METHODS**

**Chemicals and antibodies.** Buffer ingredients and substrates such as estrone-3-sulfate, forskolin, and DHEA-S were purchased from Sigma-Aldrich (Deisenhofen, Germany).

The use of affinity-purified, polyclonal rabbit anti-rat OAT3 (rOAT3; COOH-terminal peptide) antibody and the respective immunizing peptide in immunocytochemical experiments have been recently described (13). In preliminary experiments, we proved that the antibody cross-reacted with the OAT3 in human tissues (hOAT3). A reliable hOAT4-antibody was not available. The secondary antibodies, which included the CY3-labeled (GARCY3) or alkaline phosphatase-labeled (GAP) goat anti-rabbit IgG were purchased from Jackson Immunoresearch Laboratories (West Grove, PA). The fluorescence fading retardant Vectashield was purchased from Vector Laboratories (Burlingame, CA).

**Cell culture and cortisol release and ^{3}H[JES uptake experiments.** Human NCI-H295R adrenal tumor cells (11) were grown in 75-cm² flasks (Falcon, Lincoln Park, NJ) at 37°C with 5% CO₂ in DMEM and Ham’s Nutrient Mixture F-12 (1:1, vol/vol) containing: 15 mM HEPES, 1% ITS + Premix (Becton-Dickinson, Heidelberg, Germany), 6.25 μg/ml insulin, 6.25 μg/ml transferrin, 6.25 ng/ml selenium, 10% bovine serum albumin, 5.35 μg/ml linoleic acid, 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mM glutaric acid. The medium was changed twice a week.

Cells used for experiments were subcultured from 80% confluent stock cultures into six-well culture plates (Nunc, Wiesbaden, Germany) ≥24 h before the start of the experiment. Each well was washed three times with 2 ml of phosphate-buffered saline (PBS) before the start of incubation. Twenty-four-hour incubations were conducted on each set of plates with 2 ml of complete DMEM-F-12 in the absence or presence of 10 μM forskolin, 0.5 mM ES, or 0.5 mM DHEA-S. At the end of the incubation period, the medium was removed from each well and kept at −20°C for cortisol measurement. The protein concentration was determined according to Bradford (6), with serum albumin as a standard.

For ES uptake experiments, NCI-H295R cells were subcultured in 24-well plates ≥48 h before the start of the uptake experiment. At the start of experiment, the cells were washed three times with minimum- 

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amplification was performed with 26–30 cycles. GAPDH primers were used to standardize the expression. An initial amplification of GAPDH and OATs was made to optimize the number of PCR cycles to remain in linear phase. Normally, 17–19 cycles were used for GAPDH and 26–30 cycles for OATs, depending on expression level to standardize the expression. An initial amplification of amplification was performed with 26–30 cycles. GAPDH primers were first incubated with medium containing 10 mM mannitol, 5 mM EGTA, 12 mM Tris-HCl, pH 7.4, for 1 h and then incubated with blotting buffer containing rOAT3 antibody (1:50) overnight in a refrigerator. Following intensive washing in the antibody-free blotting buffer, the transfer membrane was further incubated for 1 h in the blotting buffer that contained 0.1 mg/ml GARAP, washed four times with blotting buffer, and stained for alkaline phosphatase activity with the BCIP/NBT method. The stained membranes were scanned using an Epson scanner.

To demonstrate nonspecific labeling, the rOAT3 antibody was preincubated with the immunizing peptide (final concentration of the peptide 0.5 mg/ml) for 4 h at room temperature and then used in immunoblotting assay as described above.

**Immunocytochemistry.** The semiconfluent cells from the flasks were seeded on sterile 1-cm round glass coverslips and placed in 6-well culture plates 48 h before the start of experiment. To determine the effect of forskolin and DHEA-S stimulation on the expression of OATs, the cells were stimulated with medium containing 10 μM forskolin, 100 μM DHEA-S, or without any stimulation as a control, for 24 h. At the end of stimulation, cells were washed three times with PBS at room temperature and incubated with freshly prepared 4% p-formaldehyde in PBS at 4°C for 30 min, followed by washing (4 × 5 min) in PBS. To expose hOAT3 binding domains for the antibody, we employed an antigen retrieval technique. The fixed and washed cells were immersed in 10 mM citrate buffer, pH 3, heated in a microwave oven at 800 W for 10 min, and then cooled down to room temperature in the same buffer for another 20 min. Then followed (steps) washing in PBS (3 × 5 min), incubation in 0.5% Triton X-100 (in PBS, 15 min) and 2% Triton X-100 (in PBS, 30 min), washing in PBS (2 × 5 min), incubation in 1% bovine serum albumin (in PBS, 30 min) and rOAT3-antibody (1:100 in PBS, overnight at 4°C), washing in PBS (4 × 5 min), incubation in GARCY3 (1.6 mg/ml) at room temperature for 1 h, and washing in 0.5% Triton X-100 (in PBS, 2 × 10 min) and in PBS (1 × 10 min). The samples were then covered with Vectashield and prepared for the fluorescence inspection.

To demonstrate specific staining, the rOAT3 antibody was preincubated with the immunizing peptide (final concentration of the peptide 0.5 mg/ml) for 4 h at room temperature and then used in immunofluorescence assay as described above.

The fluorescence was examined and photographed with an Opton III RS fluorescence microscope (Opton Feintechnik, Oberkochen, Germany) using a Spot RT Slider camera and software (Diagnostic Instruments, Sterling Heights, MI). The photos were imported into Adobe Photoshop 6.0 and processed and labeled as required.

**Statistics.** Immunofluorescence pictures represent findings in three independent experiments. The numeric data are presented as means ± SE, with the release of cortisol under a standard condition being set at 100% in each experiment. Statistical analysis was performed by Student’s t-test for unpaired data.

**RESULTS**

Forskolin, DHEA-S, and ES induced cortisol release from NCI-H295R cells. In previous results from RT-PCR studies, we showed the presence of hOAT3 and hOAT4 in the NCI-H295R cells. In addition, we demonstrated a fourfold greater cortisol uptake by hOAT3-expressing oocytes and a saturable radiolabeled ES uptake in NCI-H295R cells (1). Because ES and DHEA-S are substrates of hOAT3, hOAT4, and OATP family members, we conducted a comparative study to evaluate the effect of forskolin, DHEA-S, and ES on the cortisol release from NCI-H295R cells. The cells grown in six-well plates were incubated in medium with or without 10 μM forskolin or 0.5 mM DHEA-S or 0.5 mM ES for 24 h. At the end of a 24-h incubation period, medium samples were collected to determine the effect of the test substances on the efficiency of the transfer. After destaining, the membrane was first blocked by incubating in the blotting buffer (5% nonfat dry milk, 0.15 M NaCl, 0.02% Na2SO3, 1% Triton X-100, 20 mM Tris-HCl, pH 7.4) for 1 h and then incubated with blotting buffer containing rOAT3 antibody (1:50) overnight in a refrigerator. Following intensive washing in the antibody-free blotting buffer, the transfer membrane was further incubated for 1 h in the blotting buffer that contained 0.1 mg/ml GARAP, washed four times with blotting buffer, and stained for alkaline phosphatase activity with the BCIP/NBT method. The stained membranes were scanned using an Epson scanner.

To demonstrate nonspecific labeling, the rOAT3 antibody was preincubated with the immunizing peptide (final concentration of the peptide 0.5 mg/ml) for 4 h at room temperature and then used in immunoblotting assay as described above.

![immunoblot](image-url)
cortisol release. The results were standardized to picomol of cortisol per milligram of protein by measuring the protein concentration of each well. As shown in Fig. 1, 24-h forskolin treatment (10 μM) of NCI-H295R cells increased the cortisol secretion by more than 25-fold compared with nonstimulated control cells. ES treatment did not cause any change in cortisol release, whereas the cells incubated with DHEA-S revealed a 10-fold greater cortisol release than control cells.

When DHEA-S was incubated simultaneously with forskolin, the cortisol release was higher than in the cells treated only with forskolin (Fig. 2). In this setting, the control cells (without forskolin and DHEA-S treatment) showed only a very small cortisol release compared with the forskolin-treated cells. However, the cortisol release in the presence of forskolin and 0.05 or 0.5 mM DHEA-S was significantly higher than that with forskolin alone. These results indicate that DHEA-S had an additive stimulative effect on the forskolin-induced cortisol release (Fig. 2).

Effect of forskolin and DHEA-S on mRNA expression for key enzymes of steroid biosynthesis. Because forskolin and DHEA-S increased cortisol production, we examined the effect of forskolin and DHEA-S on mRNA expression for key enzymes of steroid biosynthesis. The specific primers for StAR, side-chain cleavage enzyme desmolase (CYP11A1), 17α-hydroxylase/17,20-lyase (CYP17), 3β-HSD, 21-hydroxylase (CYP21A2), and steroid 11β-hydroxylase (CYP11B1) were used to amplify cDNA of nonstimulated control cells and of forskolin- or DHEA-S-stimulated cells. GAPDH was taken as a reference from the same cDNA to standardize and compare the results.

Figure 3A shows a representative RT-PCR. The densitometric data (Fig. 3B) of the RT-PCR after 24-h stimulation with forskolin showed increased mRNA expression of StAR by 25 ± 3.0% (P < 0.0001), CYP11A1 by 13 ± 4.0% (P < 0.04), CYP17 by 45 ± 9.0% (P < 0.003), 3β-HSD by 80 ± 5.0%

(P < 0.0001), CYP21A2 by 55 ± 6.0% (P < 0.0001), and CYP11B1 by 80 ± 30% (P < 0.08). DHEA-S stimulation revealed a significant increase in the expression for StAR by 15 ± 3.0% (P < 0.03), but no significant change was observed in mRNA expression of CYP11A1, CYP17, 3β-HSD, and CYP21A2 in DHEA-S-treated NCI-H295R cells. CYP11B1 was increased by 108 ± 70%, but, due to the large scatter of the data, this increase was statistically not significant.

Effect of forskolin and DHEA-S treatment on mRNA expression for hOAT3 and hOAT4. The mRNA expression for hOAT3 and hOAT4 was evaluated by RT-PCR in nonstimulated controls as well as in forskolin- and DHEA-S-stimulated cells. GAPDH was used as reference from the same cDNA to standardize and compare the results (Fig. 4A). The results shown in Fig. 4B demonstrate increases (21.1 ± 10.0%, P < 0.15, and 53.3 ± 32%, P < 0.07) of hOAT3 mRNA expression following the forskolin and DHEA-S treatments, respectively. The mRNA expression for OAT4 remained unchanged; with forskolin, however, there was a 39 ± 7.5% (P < 0.002) increase by DHEA-S.

Immunoblotting of hOAT3. We next used the rOAT3 antibody to test the expression of hOAT3 protein by immunoblotting of isolated cell membrane fractions. In Western blots of total cell membrane isolated from the control, forskolin-, and DHEA-S-treated cells, the rOAT3 antibody labeled multiple protein bands (Fig. 5A). Three clear-cut bands of 90 kDa and above were upregulated in forskolin- and DHEA-S-treated cells. Following preincubation of the antibody with the immunizing peptide, these bands were completely blocked (Fig. 5B). The densitometry measurement of the 90-kDa proteins on the Western blot showed an increase of hOAT3 expression by forskolin and DHEA-S treatment to 257 ± 70% (P < 0.05) and by DHEA-S to 342 ± 156% (P < 0.15), respectively, compared with untreated control cells. β-Actin expression was used as a control of similar protein load and showed no change in control or treated cells. These data suggest a comparable
pattern of expression of OAT3 protein, its mRNA, and the cortisol release in NCI-H295R cells treated with forskolin and DHEA-S.

**Immunocytochemistry of hOAT3.** Finally, we used the rOAT3 antibody to show the expression of hOAT3 protein by immunostaining. The control NCI-H295R cells were only weakly stained with the antibody (Fig. 6A, left). The staining was largely abolished in the cells following peptide-block of the antibody (Fig. 6A, middle), and it was not different from the background staining in the absence of the primary antibody (Fig. 6A, right). The staining was increased in the forskolin-treated cells (Fig. 6B, left); even at a 40-fold shorter exposure time (0.5-s compared with 20-s exposure in nonstimulated control cells, top) the staining appeared brighter, indicating a strong increase in OAT3 labeling by forskolin treatment. This staining was completely blocked by the peptide-saturated an-
positive staining, it was not possible to clearly assign the staining to the plasma membrane and/or to intracellular organelles and cytoplasm. Many cells exhibited an overall strong but granular intracellular staining (details not shown), indicating that some unidentified intracellular organelles might contain OAT3 protein in a higher abundance than the cell membrane and the surrounding cytoplasm. DHEA-S stimulated ES uptake in adrenal cells. For functional validation of OAT-mediated [3H]ES uptake, we incubated confluent adrenal cells with radioactively labeled ES for 1 min. To test for the influence of DHEA-S on the OAT3-mediated ES uptake, the cells were preincubated with 100 μM DHEA-S for 24 h. As shown in Fig. 7, preincubation with DHEA-S led to a fivefold stimulation of ES uptake compared with nonstimulated control cells. [3H]ES uptake was cis-inhibited by 100 μM unlabeled ES. These results confirmed functionally the elevation of OAT3 expression in NCI-H295R cells as observed in immunostaining.

**DISCUSSION**

Cortisol is believed to cross cellular membranes solely by simple diffusion. Although this anticipation may hold for most cells, rapid release from cortisol-producing adrenal cells or substantial transepithelial transport, e.g., in renal proximal tubules, may require the presence of transport systems. Indeed, earlier experiments on bovine adrenal cells in primary culture provided evidence for the involvement of transporters for organic anions in the release of cortisol. The inhibitor of OATs, probenecid decreased cortisol release, and extracellular PAH and glutarate increased it by trans-stimulation (17, 18). Bovine adrenal cells exhibited probenecid-sensitive PAH uptake that was inhibited by cortisol (18), indicating a transporter common for PAH, glutarate, and cortisol. In rat adrenal glands, OAT1 was localized by immunocytochemistry to the zona fasciculata cells and was shown to increase in amount after stimulation by ACTH (2). In the human adrenal cell line NCI-H295R, cortisol release was inhibited by 24-h preincubation with probenecid, PAH, glutarate, and cimetidine (1). The uptake of labeled ES and PAH was trans-stimulated by preloading the cells with glutarate, PAH, or cortisol, suggesting again the involvement of an OAT. RT-PCR experiments revealed the expression of OAT3 and OAT4. OAT3, but not OAT4, was able to translocate labeled cortisol (1). Taken together, these data provided evidence for a physiological role of OAT1 (in bovine and rat adrenals) or OAT3 (in human adrenal cells) in cortisol release.

During our experiments on the influence of high-affinity substrates of OAT3 on cortisol release, we observed that DHEA-S, but not ES, stimulated basal cortisol release from NCI-H295R cells in the absence of forskolin. An increased release may be due to trans-stimulation, with extracellular DHEA-S exchanging for intracellular cortisol through OAT3 at the plasma membrane. However, two lines of evidence argue against trans-stimulation as the underlying mechanism. First, ES is transported by human OAT3 similarly to DHEA-S (7) but failed to stimulate cortisol release. Second, NCI-H295R cells were preincubated for 24 h with the steroid sulfates, leading to their complete equilibration across the cell membrane. In this setting, a trans-stimulation by extracellular DHEA-S should be blunted by cis-inhibition by intracellular DHEA-S. Therefore, since trans-stimulation is an unlikely

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**Fig. 5.** Immunoblotting of total cell lysate from NCI-H295R cells for hOAT3 protein. Cells were incubated with medium containing 10 μM forskolin, 100 μM DHEA-S, or control without stimulus for 24 h, washed, and dissolved in lysis buffer. Equal amounts of protein [total cell membrane (TCM)] were loaded into each lane and reacted with the COOH-terminal rat (r)OAT3 polyclonal antibody (A) and the COOH-terminal rOAT3 polyclonal antibody saturated with peptide (antigen) (B). The figure depicts the result of 1 representative experiment. C: the relative intensity (%control) was measured by densitometry from 3 independent experiments with different batches of TCM. Data points are means ± SE, and significance was determined by Student’s t-test against control cells (*P < 0.05).
explanation for increased cortisol release, we evaluated a possible effect of DHEA-S on key enzymes of cortisol synthesis by using forskolin as a reference.

Using semiquantitative RT-PCR, we observed after 24-h forskolin treatment of NCI-H295R cells a highly significant (80%) increase in the expression of 3β-HSD. The mRNAs for StAR, CYP21A2, CYP17, and CYP11A1 were increased by 25, 55, 45, and 13%, respectively. Forskolin also increased the expression of CYP11B1 by ~80%, but this increase appeared to be nonsignificant due to high variability of the individual data. These results are in a good agreement with previously reported effects of forskolin stimulation in primary adrenal as well as in NCI-H295R cells (3, 5, 9, 15). Stimulation of NCI-H295R cells with forskolin induced a 1.5-fold increase in StAR mRNA (21). Bird et al. (4) reported an increase in the expression of CYP21A2, CYP17, CYP11A1, CYP11B1, and CYP11B2 enzymes by forskolin as well as by dibutyryl-cAMP.

Following DHEA-S treatment, a slight, but significant, increase in the StAR mRNA was observed. The StAR protein is involved in cholesterol translocation from the cytosol into the mitochondria and serves as a rate-limiting step in steroid biosynthesis. There was a twofold, albeit not significant increase in the mRNA of CYP11B1, the last key enzyme in

Fig. 6. Immunostaining of OAT3 in control and forskolin- and DHEA-S-treated NCI-H295R cells. Cells were grown for 24 h on 1-cm Ø slides in medium without stimulus (A) or in medium containing 10 μM forskolin (B) or 100 μM DHEA-S (C). Immunostaining for OAT3 localization was done with the COOH-terminal rat OAT3 antibody (left) and antibody saturated with antigen peptide (middle), and only secondary antibody was applied for unspecific staining (right). Immunostaining was documented at the exposure time indicated on the slides. The figure depicts the result of 1 representative experiment. Similar results were obtained in 4 additional experiments with different passages of cells.

Fig. 7. [3H]ES uptake by DHEA-S-stimulated NCI-H295R cells. Cells were grown in 24-well plates ±24 h before the start of the experiment. Cells were preincubated with or without 100 μM DHEA-S for a further 24 h. After the incubation time, medium was removed and washed with Ringer buffer. For uptake, cells were incubated with [3H]ES (0.44 Ci/ml, 10 nM) in Ringer solution for 1 min. [3H]ES content in cells was determined and normalized to the protein concentration and presented in fmol/mg protein. Data points are means ± SE of 4 single determination data points. The figure represents 3 different experiments with 4 single data points.
cortisol biosynthesis, which converts 11-deoxycortisol to cortisol. The expression of other tested enzymes was not changed by DHEA-S in NCI-H295R cells. Chang et al. (8) recently reported no change in mRNA expression of CYP11A1 and StAR by DHEA-S treatment in rat zona fasciculata-reticularis cells. The protein expression for StAR was even decreased by DHEA-S treatment, whereas CYP11A1 protein remained unchanged. At present, we do not know whether there are species differences (humans vs. rats) or differences between cell lines and intact tissue.

In this study, cortisol release from NCI-H295R cells was increased 25-fold by pretreatment with forskolin and 10-fold by preincubation with DHEA-S. The lower efficiency of DHEA-S may be reflected by its sole effect on the expression of StAR and, possibly, on CYP11B1, whereas forskolin upregulated the expression of StAR, 3β-HSD, CYP21A2, CYP17, and CYP11A1. Nevertheless, the relative increase in mRNA (up to 80% at most) lagged far behind the increase in cortisol release (2,500%), suggesting a further regulatory effect, e.g., on OATs. The expression of OAT3 was increased by forskolin and DHEA-S, albeit to a small extent. OAT4, which does not translocate cortisol (1), was slightly more expressed in forskolin and DHEA-S, albeit to a small extent. DHEA-S treatment, whereas CYP11A1 protein remained unchanged. At present, we do not know whether there are species differences (humans vs. rats) or differences between cell lines and intact tissue.

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In conclusion, forskolin and, to a lesser extent, DHEA-S increase cortisol release from the human adrenal cell line NCI-H295R. The stimulation of hormone release is accompanied by a relatively small increase in expression of key enzymes of steroid biosynthesis, but a large increase in OAT3 expression and transporter-mediated ES uptake. This finding provides further evidence for a physiological role of OAT3 in cortisol production and release by human adrenal cells.


