Dexamethasone Suppresses Neurosteroid Biosynthesis via Downregulation of Steroidogenic Enzyme Gene Expression in Human Glioma GI-1 Cells

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Emerging evidence indicates that stress hormone glucocorticoids (GC) are an important modulator of brain development and function. To investigate whether GCs modulate neurosteroid biosynthesis in neural cells, we studied the effects of GCs on steroidogenic gene expression in human glioma GI-1 cells. The GC dexamethasone (Dex) reduced steroidogenic acute regulatory protein (StAR), CYP11A1 and 3β-hydroxysteroid dehydrogenase gene expression in a dose- and GC receptor-dependent manner. In addition to its effects on steroidogenic gene expression, Dex also reduced de novo synthesis of progesterone (PROG). Furthermore, Dex inhibited all-trans retinoic acid (ATRA) and vitamin D3-induced steroidogenic gene expression and PROG production. This suggests that GC regulates steroidogenic gene expression in neural cells via cross-talk with the two fat-soluble vitamins, A and D. The relationship between the effects of GCs on neurosteroid biosynthesis and on cognitive behaviors and hippocampal neural activity is also discussed herein.

Key words CYP11A1; glia; dexamethasone; neurosteroid; progesterone; steroidogenic acute regulatory protein (StAR)

Glucocorticoids (GCs) are widely used as drugs to treat inflammatory and autoimmune disorders, including neuroinflammatory conditions such as multiple sclerosis. In response to an inflammatory reaction or to stress, the hypothalamic–pituitary–adrenal (HPA) axis is stimulated to increase systemic levels of glucocorticoids with a consequent repression of inflammation in a process involving nuclear factor kappa B (NF-κB). Many studies have described the effects of GCs on the central nervous system (CNS). GCs activate several biochemical/molecular processes in the hippocampus through two receptors, the glucocorticoid receptor (GR) and the mineralocorticoid receptor (MR). GCs influence cognitive behaviors and hippocampal neural activity, and also increase the rate of aging-dependent cell loss in the hippocampus. During emotional and stressful situations, activation of the HPA axis causes the adrenal cortex to release GCs, which travel through the bloodstream and cross the blood–brain barrier to activate GRs throughout the brain. Steroids that are synthesized within the central or peripheral nervous systems are termed “neurosteroids.” The nervous system is an important site of steroid production, as both neurons and glial cells can synthesize steroids de novo from cholesterol. To start to synthesize steroids, cholesterol is needed to transport into the mitochondria, mediated by the action of steroidogenic acute regulatory protein (StAR). StAR is a mitochondrial protein that is rapidly synthesized in response to stimulation of the cell to produce steroid. The mitochondrial cytochrome P450scc (CYP11A1), which is the cholesterol side-chain cleavage enzyme that catalyzes the de novo synthesis of pregnenolone (PREG), is expressed throughout the rodent brain. 3β-Hydroxysteroid dehydrogenase (HSD3B1), which converts PREG to progesterone (PROG), is also largely distributed throughout the brain and spinal cord. In addition, primary cultures of mixed glial cells can metabolize cholesterol to PREG and PROG. Neurosteroids are involved in the regulation of several CNS processes, specifically mood, affective and cognitive functions. Elevated circulating levels of glucocorticoids (GCs) are associated with psychiatric symptoms such as depression and dementia, although it remains unknown if this is a cause or an effect of the psychiatric condition. In addition, depression is a well-known side effect of glucocorticoid treatment, and a third of the patients receiving glucocorticoids experience significant mood disturbances and sleep disruption. However, GC effects on neurosteroid synthesis have not been well documented.

Previously, we reported that vitamin A and vitamin D (VD) induce neurosteroid biosynthesis in human glioma GI-1 cells through the induction of steroidogenic gene expression. To investigate the effects of GCs on neurosteroid biosynthesis in GI-1 cells, we analyzed the effect of dexamethasone (Dex) on steroidogenic gene expression within these cells.

MATERIALS AND METHODS

Reagents 1α,25-Dihydroxy vitamin D3 (VD3) was purchased from Cayman Chemical Company (Ann Arbor, MI, U.S.A.). All-trans retinoic acid (ATRA) and dexamethasone (Dex) were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Pregnenolone (PREG) and progesterone (PROG) were obtained from Sigma (St. Louis, MO, U.S.A.). GI-1 cells were obtained from the Riken cell bank (Tsukuba, Japan). Anti-HSD3B1 antibody and peroxidase-conjugated secondary antibody were purchased from Abcam (Danvers, MA, U.S.A.) and Dako (Glostrup, Denmark), respectively.

Cell Culture GI-1 is a human glioma cell line established from a tumor specimen removed from the left frontoparietal region of a 61-year-old man. Cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented

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with 10% fetal bovine serum (FBS), 10 U/mL penicillin and 10 U/mL streptomycin at 37°C in a humidified atmosphere of 5% CO₂. For ATRA/VD₃ or Dex treatments, exponentially growing cells were split into 6-well plates at 3×10⁵ cells/well and cultured for 4–5 d in medium supplemented with 10% FBS. This growth medium was replaced with DMEM containing 5% charcoal-treated serum and the indicated treatments (vitamins and Dex) at various concentrations in 0.1% DMSO.

**Reverse Transcription Polymerase Chain Reaction (RT-PCR)** Total RNA was isolated using a guanidium thiocyanate phenol-chloroform extraction method. First-strand cDNA was synthesized from 5µg of total RNA using 100 units of reverse transcriptase (ReverTra Ace, TOYOBO, Tokyo, Japan) and random primers, according to the manufacturer’s protocol. PCR was then performed, using this synthesized cDNA as a template, with Taq polymerase (GoTaq, Promega, Madison, WI, U.S.A.). Amplification was performed using 30 cycles of 1 min at 95°C, 1 min at 58°C, and 1 min at 72°C. Quantitative real-time PCR was performed using an ABI-Prism 7300 thermal cycler and a SYBR green PCR reagent kit (Roche Diagnostics K. K., Tokyo, Japan). Ct (cycle at which threshold fluorescence is reached) values for each sample were then collected at a threshold level of fluorescence set within the linear phase of amplification. Calculations of the initial amounts of mRNA were performed according to the cycle threshold method.³⁷ The mRNA levels were normalized using the 18S ribosomal RNA (rRNA) levels, which had been quantified by real-time PCR. PCR primers used to amplify the steroidogenic cDNAs were designed from published DNA sequences using Primer Express version 3.0 (Applied Biosystems, Foster City, CA, U.S.A.). The sequences of the primers used are as follows: 18S rRNA: forward 5'-TGG TTG CAA AGC TGA AAC TTA AAG-3' and reverse 5'-AGT CAA ATT AAG CCG CAG GC-3'; StAR, forward 5'-CCA CCC CTA GCA CGT GGA T-3' and reverse 5'-ATT GTC CTG CTG ACT CTC CTT CT-3'; CYP11A1, forward 5'-AGG AGG AGG GTG GGA CAC GAC-3' and reverse 5'-TTG CGT GCT GCC ATC TCA TAC A-3'; and HSD3B1, forward 5'-TCA TCC GCC TCT TGG TGA AG-3' and reverse 5'-AGC ACT GTC AGC TTG GTC TTG TT-3'.

**Immunoblotting** Cells were harvested in ice-cold phosphate buffered saline (PBS) and lysed in Nonidet P-40 lysis buffer (50 mM Tris–HCl, pH 8.0, 120 mM NaCl, 1 mM ethylenediaminetetraacetic acid (EDTA) pH 8.0, 0.5% Nonidet P-40, 10 mM β-glycerophosphate, 2.5 mM NaF, 0.1 mM Na₃VO₄) supplemented with protease inhibitors. Denatured samples were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride membranes (Millipore, Billerica, MA, U.S.A.). Membranes were probed using the designated antibodies and visualized with the ECL detection system (GE Healthcare, Little Chalfont, United Kingdom).

**Radiolabeling of Steroid Hormones and Analysis by Thin Layer Chromatography (TLC)** To label cholesterol...
and steroid molecules, 1 mCi/mL of [1-14C]acetic acid sodium salt (57 mCi/mmol, Moravek Biochemicals, Brea, CA, U.S.A.) was added to cultures for 24 h. The culture medium was then collected and steroids were extracted as described previously. Briefly, an equal volume of ethyl acetate–isooctane (1:1, v/v) was added to the media. This mixture was then centrifuged and the upper organic phase was collected. The extraction procedure was repeated twice, and the organic phase was dried and resuspended in 20 µL of ethyl acetate. Steroids (5 µL) were separated on silica-gel 60 F245 plates (Merck, Darmstadt, Germany) with chloroform–ethyl acetate (4:1) as the mobile phase. Radioactive lipids on the TLC plate were visualized using an FLA7000 imaging system (FUJIFILM, Tokyo, Japan). A non-radioactive standard was included on the plate and visualized by UV or phosphomolybdic acid staining. The migration of standard steroids was used to identify the radioactive steroids on the same plate.

**Statistical Analysis** Statistical significance was determined by Student’s t-tests. For multiple comparisons, data were analyzed using Dunnett’s multiple comparison test. Results were considered significant when \( p < 0.05 \).

**RESULTS**

**Effect of Dex on the Steroidogenic Gene Expression in GI-1 Cells** To examine whether GC affects neurosteroid biosynthesis in neural cells, we measured the expression of three steroidogenic genes in human glioma GI-1 cells treated with a physiological range of Dex concentrations. Cells were incubated with Dex for 24 h, and StAR, CYP11A1 and HSD3B1 gene expression was measured by real-time PCR. As shown in Fig. 1, the expression of all these genes was reduced by Dex in a dose-dependent manner. The expression of all three genes was almost 50% lower in cells treated with 10 nM Dex than in control cells. Profile of Dex-mediated reduction in StAR gene expression was different from other two genes. Half of StAR gene expression was insensitive to Dex (Fig. 1C). Immunoblot analysis also showed that Dex reduced the amounts of HDS3B1 proteins according to the levels of its gene expression (Fig. 1D). We could not measure the protein levels of StAR and CYP11A1 because of low amounts of proteins.

As shown in Fig. 2, addition of RU-486, a GR antagonist, abolished the effect of Dex on these gene expression, suggesting that Dex regulates these gene expression via GR. Interestingly, RU486 alone induced expression of three genes.

**Effects of Dex on Vitamin-Induced Steroidogenic Gene Expression in GI-1 Cells** Previously, we reported that ATRA (a form of vitamin A) and VD₃ induce neurosteroid biosynthesis in GI-1 cells through induction of steroidogenic gene expression. To elucidate whether Dex affects vitamin-induced neurosteroid synthesis in GI-1 cells, we co-treated GI-1 cells with Dex and the vitamins (ATRA and/or VD₃). As shown in Fig. 3, Dex reduced the vitamin-mediated induction of all three steroidogenic genes tested, StAR, CYP11A1 and HSD3B1. Immunoblot analyses also showed that Dex reduced the induction of HSD3B1 proteins mediated by ATRA.
and/or VD₃ (Fig. 3D).

**Dex Reduces de Novo Biosynthesis of Neurosteroids in GI-1 Cells** We next measured the de novo biosynthesis of neurosteroids in Dex-treated GI-1 cells using a previously described method. GI-1 cells were treated with 1 µM Dex for 48 h and [1-14C] acetic acid was then added to the culture medium for 24 h prior to collection of the medium. Radiolabeled steroids were extracted from culture media samples, separated by TLC, and visualized. The level of steroid production was quantified based on the band intensities, and an index of induction relative to the non-treated control was calculated (Fig. 4). Low but significant levels of pregnenolone (PREG) and progesterone (PROG) were synthesized and secreted into the culture medium. Following Dex addition, the de novo synthesis of PROG decreased to half the level of the control, whereas that of PREG did not change. Dex also reduced vitamin-induced PROG production, although it slightly increased PREG production.

**DISCUSSION**

In the present study, we described the effects of the glucocorticoid Dex on neurosteroid synthesis in the human glioma cell line, GI-1. Our results indicated that in GI-1 cells Dex reduces the de novo synthesis of neurosteroids through down-regulation of steroidogenic genes such as StAR, CYP11A1 and HSD3B1. The effect of Dex was mediated by GR, since RU-486, an antagonist of GR, abolished the downregulation of (Fig. 2).

It has been reported that in human adrenal cells Dex has no effect on steroidogenic gene expression, including the genes studied here, whereas Dex inhibits ACTH-induced activation of CYP11A1 gene expression in bovine adrenal cells. In gonadal and adrenal steroidogenesis, SF-1 is a critical transcriptional regulator; however, in the brain, SF-1 is not essential for steroidogenesis. All these data indicate that steroidogenic gene regulation differs between adrenal/gonadal and neural cells. We have previously reported that retinoic acid receptor (RAR)-retinoid X receptor (RXR) and vitamin D receptor (VDR) are key transcriptional factors for the upregulation of steroidogenic genes in GI-1 cells, whereas SF-1 is not involved. In addition, Dex reduced ATRA/VD₃-mediated induction of steroidogenic gene expression (Fig. 3). Although direct cross-talk between GR and RAR/RXR or VDR is not well documented, accumulating evidence indicates that glucocorticoids can influence the activity of other transcription factors, such as activator protein 1 (AP-1), NF-κB, and cAMP response element binding protein (CREB). In addition, several studies indicate that GR associates with other transcription factors through direct protein-protein interactions to mutually repress or stimulate each protein’s transcriptional activities. Recently glucocorticoid-mediated modulation of miRNA has been reported. Further investigation is needed to understand the mechanism underlying the regulation of steroidogenic genes by glucocorticoids in neural cells.

We found that RU468 itself increased the steroidogenic gene expression (Fig. 2). This might be due to RU468-mediated inhibition of GR action via endogenous glucocorticoids
present in culture media although we used charcoal-treated serum. This is supported by the fact that very low dose of Dex (0.001 µM) can inhibit the steroidogenic gene expression (Fig. 1).

The Dex-mediated decrease in de novo synthesis of PROG was comparable to that observed for steroidogenic gene expression (Fig. 4). However, Dex had no effect on PREG synthesis, although the expression of StAR and CYP11A1 genes was reduced to half the level of the controls (Fig. 1). One possible explanation is that reduction in the reaction catalyzed by HSD3B1 resulted in the accumulation of PREG. Furthermore, Dex-induced increases in cholesterol may reflect a reduction in cholesterol transport into mitochondria, mediated by StAR, which was downregulated by Dex (Fig. 4C).

Dex markedly reduced ATRA/VΔ3-mediated activation of steroidogenic gene expression in GI-1 cells (Fig. 3). However, the effect of Dex on the PREG production in ATRA/VΔ3-treated cells was not comparable to the expression levels of steroidogenic genes (Figs. 3, 4B), although the experimental conditions were slightly different. Dex had no effect on the PREG production in ATRA/VΔ3-treated cells. We cannot explain the reason at the moment, however, ATRA/VΔ3/Dex treatment will affect many aspects in the process of steroid production. A recent study showed post-transcriptional activation of CYP11A1 by deacetylation. Further investigation is needed to clarify this point.

In ATRA/VΔ3-treated cells, Dex slightly inhibited PROG production although it strongly reduced the HSD3B1 gene expression (Figs. 3, 4). In GI-1 cells, synthesized PROG will be catalyzed by CYP17A1 and CYP21A1. Since Dex is known to be a substrate for CYP17A1, it may compete the reaction from PROG to 17-hydroxy-PROG catalyzed by CYP17A1. This may lead to accumulation of PROG in ATRA/VΔ3/Dex-treated cells. This point should be clarified in future study.

In this report, we demonstrated that Dex reduced PROG production in human glioma GI-1 cells. PROG is a major active neurosteroid that has been reported to exert protective effects in numerous experimental models that mimic a variety of pathogenic aspects of brain dysfunction seen with advanced age or age-related neurodegenerative diseases such as Alzheimer’s disease (AD). There is growing evidence that PROG may be a safe and effective treatment for traumatic brain injury (TBI) and other neural disorders in humans. In TBI, PROG reduces edema and inflammatory cytokine production, prevents neuronal loss, and improves functional outcomes. One of the important functions of PROG in the CNS is to induce brain-derived neurotrophic factor (BDNF) expression. BDNF is a neurotrophin abundantly expressed in several areas of the CNS and is known to induce a lasting potentiation of synaptic efficacy to enhance specific learning and memory processes. Several studies have shown altered BDNF production and secretion in a variety of neurodegenerative diseases, including Alzheimer’s and Parkinson’s diseases, but also in mood disorders such as depression and schizophrenia. It is well known that stress increases GC levels in the CNS through the HPA axis and that GCs contribute to neuronal excitability and neuroplasticity, key aspects of cognitive function. However, continuously high levels of GC secretion and insensitivity to GC feedback are observed in approximately 50% of depression cases. Together with

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**Fig. 4.** Dex Reduces de Novo Biosynthesis of Neurosteroids in GI-1 Cells

(A) GI-1 cells were incubated with 1 µM Dex and [1-14C]acetic acid for 24 h. The 14C-labeled steroids in the culture medium were extracted with ethyl acetate–isooctane (1:1 v/v) and separated by TLC. TLC plates were visualized using a FLA7000 imaging system. Steroid positions were identified by reference to those of the standards. PREG, pregnenolone; PROG, progesterone; CHOL, cholesterol. (B) The intensities of the spots corresponding to CHOL, PREG and PROG were quantified using an image analyzer. Open bars, controls (0.1% DMSO); gray bars, 1 µM Dex; dotted bars, 10 µM ATRA and 100 nM VΔ3; slashed bars, 1 µM Dex, 10 µM ATRA, and 100 nM VΔ3. The results are presented using arbitrary units, with the control values set at 1. Values are the mean ± standard deviation (S.D.) of three independent experiments (n=3). *p<0.05 indicates a significant difference compared to the negative control (DMSO). **p<0.01 (n=3). (C) Schematic pathway of neurosteroid synthesis from cholesterol (CHOL) to progesterone (PROG). Dotted line represents mitochondrial outer membrane.
our results, we hypothesize that stress-induced elevated GC downregulates neurosteroid production, especially PROG, thereby leading to downregulation of BDNF expression. Unfortunately, as GI-1 cells do not express PROG receptors, it is not possible to elucidate this hypothesis using GI-1 cells. Thus further investigation is required using other cell lines.

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