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ORIGINAL PAPER

Dexamethasone-induced suppression of steroidogenic acute regulatory protein gene expression in mouse Y-1 adrenocortical cells is associated with reduced histone H3 acetylation

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Abstract In this study, we investigated the effect of dexamethasone on the expression of steroidogenic acute regulatory protein (StAR) and the acetylation of histone H3 in mouse Y-1 adrenocortical tumor cells. Treatment of Y-1 cells with increasing concentrations (0.001–50 μg/ml) of dexamethasone for 24 h suppressed 8-Br-cAMP (0.5 mM)-stimulated StAR mRNA and protein levels and progesterone production in a dose-dependent manner. Treatment of Y-1 cells with 8-Br-cAMP (0.5 mM) for 1–24 h resulted in a marked increase in StAR mRNA levels. This increase was associated with an increase in progesterone production. StAR mRNA was down-regulated by dexamethasone at times greater than 3 h. To evaluate dexamethasone effect on the endogenous StAR gene, chromatin immunoprecipitation assays were performed in combination with polymerase chain reaction. 8-Br-cAMP increased histone H3 acetylation within the proximal region of the StAR gene promoter and coincubation with dexamethasone blocked this effect. Dexamethasone had no effect on glucocorticoid receptor mRNA expression. These results demonstrate that dexamethasone repression of 8-Br-cAMP-stimulated StAR gene expression in Y-1 cells is accompanied by reductions in histone H3 acetylation associated with the StAR gene promoter.

Keywords Mouse Y-1 adrenocortical cells · Dexamethasone · Steroidogenic acute regulatory protein · Corticosteroidogenesis · Histone H3 acetylation

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Introduction

Evidence from studies in vivo [1] and in vitro [2] suggests that the adrenal cortex is capable of self-suppression. Experiments in vivo show that raising the plasma glucocorticoid concentration either by administration of glucocorticoid [3] or by stressing the animal [4] reduces the capacity of the adrenal cortex to secrete glucocorticoids. Experiments in vitro using adrenal gland tissue [5], freshly isolated adrenocortical cells incubated briefly [6] and cultured fetal [7], adult [8], and tumorous [9] adrenocortical cells show that a variety of corticoids can suppress adrenocortical function.

It has been shown that acute self-suppression of corticosteroidogenesis in isolated adrenocortical cells is at least partly mediated by the degradation of end-product glucocorticoids [10]. This degradation occurs through an increase in adrenal 5α-reductase activity [11], an enzyme that degrades corticosterone to 5α-dihydrocorticosterone and 3β,5α-tetrahydrocoticosterone, and 11β-hydroxysteroid dehydrogenase activity [12], an enzyme that catalyzes the conversion of corticosterone to 11-dehydrocorticosterone. The presence of glucocorticoid receptor (GR) in the adrenal cortex [13] provides additional evidence for a direct glucocorticoid suppressive effect on the adrenal gland. Dexamethasone was found to inhibit the corticotropin (ACTH)-induced accumulation of cytochrome P450 side-chain cleavage (P450scc) and cytochrome P450 17α-hydroxylase (P450c17) mRNAs at a transcription level in bovine adrenocortical cells in primary culture [14]. The inhibitory effect of dexamethasone is mediated by the GR. Thus, the direct actions of glucocorticoids on the corticosteroid-producing cells of the adrenal gland may contribute to the adrenal suppression seen with therapeutic glucocorticoid administration. A short feedback loop may exist, thereby providing an additional regulatory pathway modulating the rate of glucocorticoids produced.

The biosynthesis of steroid hormones—glucocorticoids, mineralocorticoids, progesterone, estrogens, and androgens—begins from cholesterol. The rate limiting step and the main site for regulation by physiological stimuli in the activation of steroidogenesis is the delivery of cholesterol from the mitochondrial outer membrane to the inner membrane where the P450scc enzyme is located [15]. The transport of cholesterol in steroidogenic cells is thought to be mediated by steroidogenic acute regulatory protein (StAR) [16]. The strongest evidence for the critical role of StAR in regulating steroidogenesis has been demonstrated in patients suffering from congenital lipoid adrenal hyperplasia, in which both adrenal and gonadal steroid biosynthesis are markedly impaired due to mutations in the StAR gene [17]. An essentially identical phenotype is observed in StAR null mice [18]. The StAR has been identified as a phosphoprotein [19] that is rapidly induced by tropic hormones, such as gonadotropins and ACTH [20, 21]. StAR expression and regulation by tropic hormones has been examined in great detail [21]. Thus, the regulation of StAR expression would then be a key mechanism in the regulation of steroidogenesis.

Gene transcription is regulated by acetylation and deacetylation of histones [22]. Eukaryotic transcription is a highly regulated process, and acetylation is now known to play a major role in this regulation. DNA typically exists in vivo as a repeating array of nucleosomes, in which 146 bp of DNA are around a histone octamer that consists of two each of histone protein H2A, H2B, H3, and H4. This chromatin structure could be affected by histone acetylation. Histone acetylation eliminates the positive charge of ε-amino acid group on lysine residue, which could lead to destabilization and consequent dissociation of nucleosomes, thus allowing access of transcription factors and RNA polymerase to the DNA to promote transcription. Thus, histone acetylation could be thought of as an excellent marker of gene activity. Christenson et al. [23] first demonstrated that cAMP-stimulation of StAR gene transcription in MA-10 Leydig cells was associated with acetylation of histone H3 specifically within the proximal region of the StAR gene promoter.

The purpose of this study was to assess the suppressive effect of dexamethasone on StAR gene expression and progesterone production in mouse Y-1 adrenocortical cells and to elucidate the mechanism underlying its suppressive effect.

Results

Effect of treatment with dexamethasone on the expression of StAR mRNA and protein and the production of progesterone

To investigate the effects of dexamethasone on the expression of StAR mRNA and protein, Y-1 cells were incubated for 24 h with or without increasing concentrations (0.001–50 μg/ml) of dexamethasone in the presence of 8-Br-cAMP (0.5 mM). As shown in Fig. 1, dexamethasone inhibited in a dose-dependent manner 8-Br-cAMP-induced mRNA levels through Northern blot analysis (mRNA size: 3.4 kb, 1.6 kb), with the minimal effective dose at 0.001 μg/ml. The inhibition of mRNA level increased with the dose, up to a maximum of 1 μg/ml. The major 3.4 kb transcript was quantified.

Results in Fig. 2a show that dexamethasone had a significant ($P < 0.05$) effect in suppressing 8-Br-cAMP-induced StAR protein levels. The inhibitory effect was concentration dependent, and a 24-h exposure of Y-1 cells to dexamethasone concentrations ranging from 0.001 μg/ml to 10 μg/ml decreased StAR protein levels by 20–71%.

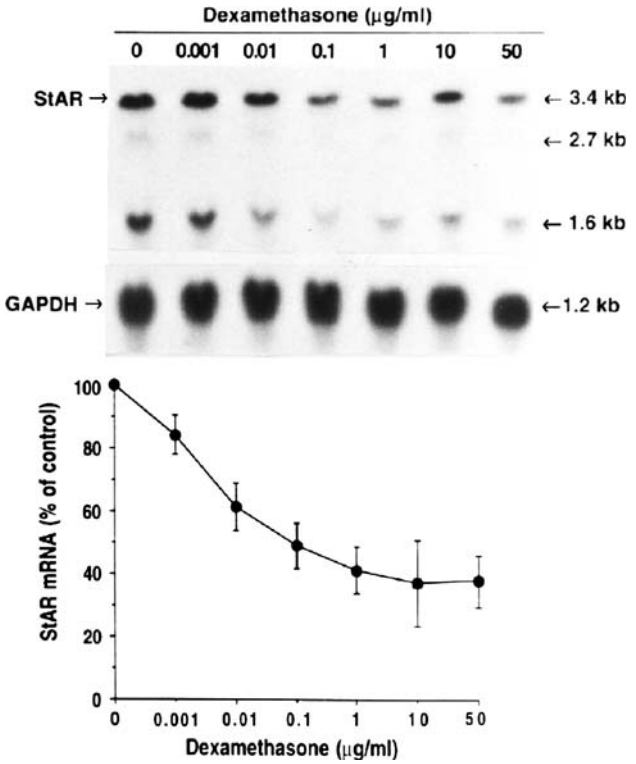

Fig. 1 Effect of dexamethasone on 8-Br-cAMP-induced StAR mRNA levels. Y-1 cells were incubated with the indicated concentration of dexamethasone in the presence of 8-Br-cAMP (0.5 mM) for 24 h. Total RNA was isolated and 25 μg from each sample were analyzed for StAR mRNA by Northern blot analysis. The positions of the 3.4- and 1.6-kilobase (kb) StAR mRNA and 1.2-kb GAPDH mRNA are indicated. Hybridization signals were quantitated using the Arcus II computer-assisted image system. Integrated optical density values for the 3.4-kb transcript of StAR were normalized to the GAPDH mRNA levels. The normalized values for the 3.4-kb StAR mRNA transcript were expressed as percent of StAR mRNA content of control cells (dexamethasone = 0). Results are the mean ± SEM of two separate experiments. Within each experiment, there were two replicates per treatment. $P < 0.05$ between control and concentration above 0.001 μg/ml. Upper panel, a representative Northern blot is shown

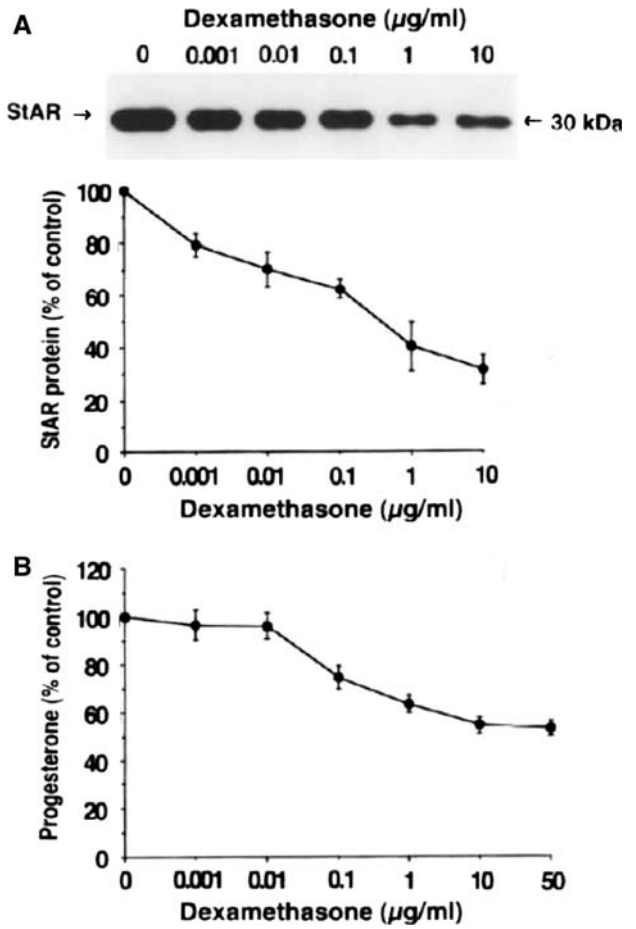

Fig. 2 Effect of dexamethasone on 8-Br-cAMP-induced StAR protein levels (**a**) and progesterone production (**b**). Y-1 cells were incubated with the indicated concentration of dexamethasone in the presence of 8-Br-cAMP (0.5 mM) for 24 h. Mitochondrial protein extracts were dose-dependently prepared and 50 μg from each sample were analyzed for StAR protein by Western blot analysis. The position of the StAR protein is indicated. The StAR protein was detected by chemiluminescence and quantitated using the Arcus II computer-assisted image system. After quantitation, integrated optical density values were expressed as percent of StAR protein content of control cells (dexamethasone = 0). Results are the mean ± SEM of two separate experiments. Within each experiment, there were two replicates per treatment. $P < 0.05$ between control and each dose tested. Upper panel, a representative Western blot is shown. The medium content of progesterone was measured by RIA. Values of progesterone production (ng/dish) were expressed as percent of progesterone production of control cells (dexamethasone = 0). Results are the mean ± SEM of two separate experiments. Within each experiment, there were two replicates per treatment. $P < 0.05$ between control and concentration above 0.01 μg/ml

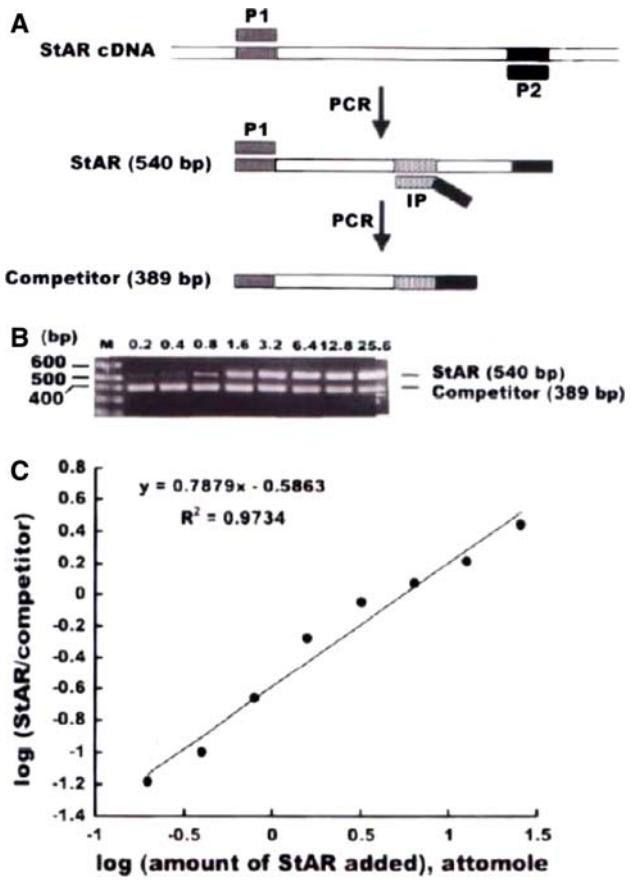

Fig. 3 Schematic representation of the preparation of StAR and competitor DNA standards for the standard curve QC-RT-PCR (**a**) and production of standard curve for quantification of StAR mRNA (**b** and **c**). Two fentomole of StAR RNA were reverse transcribed. A 2-fold serial dilution of StAR cDNA (25.6–0.2 attomole) was PCR amplified in the presence of 2-attomole competitor cDNA. The band intensity was quantified by AlphaImager computer software and used to construct standard curve

Results in Fig. 2b show that dexamethasone at a dose of 0.1 μg/ml or higher significantly ($P < 0.05$) decreased 8-Br-cAMP-induced progesterone production. The inhibited level was about 26–48% of that treated with 8-Br-cAMP alone.

In the time-course experiment, Y-1 cells were cultured with or without a constant dose of dexamethasone (10 μg/ml) in the absence or presence of 8-Br-cAMP (0.5 mM) for 1, 3, 6, 12, or 24 h. Quantitative, competitive-RT-PCR analysis (Fig. 3) revealed that StAR mRNA was down-regulated by dexamethasone at times greater than 3 h (Fig. 4a). At 6, 12, and 24 h of incubation, the amounts of 8-Br-cAMP-stimulated StAR mRNA were 2,880 ± 137, 4,050 ± 292, and 6,885 ± 125 amole/μg total RNA, respectively and were significantly decreased by treatment with dexamethasone (1,492 ± 458, 2,815 ± 791, and 4,390 ± 431 amole/μg total RNA, respectively). Results in Fig. 4b show that dexamethasone caused significant ($P < 0.05$) decreases in 8-Br-cAMP-induced progesterone production at times greater than 6 h. At 12 and 24 h of incubation, the amounts of 8-Br-cAMP-induced progesterone production were 42.3 ± 4.1 and 58.9 ± 8.0 ng/dish, respectively and were significantly decreased by treatment with dexamethasone (33.1 ± 2.0 and 42.5 ± 3.0 ng/dish, respectively).

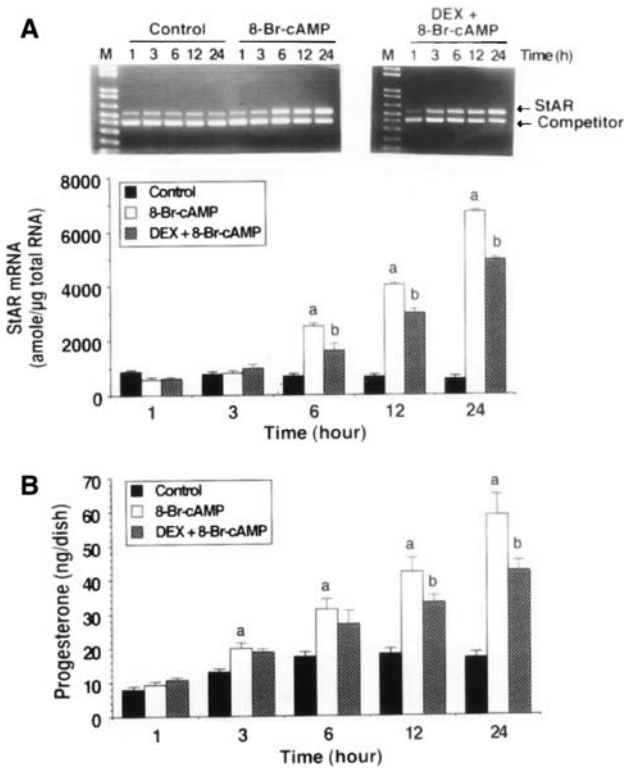

Fig. 4 Time-dependent effect of dexamethasone (DEX) on 8-Br-cAMP-induced StAR mRNA levels (**a**) and progesterone production (**b**). Upper panel, the representative data on QC-RT-PCR using the total RNA isolated from Y-1 cells treated with or without (control) DEX (10 μg/ml) in the presence or absence (control) of 8-Br-cAMP (0.5 mM) for 1, 3, 6, 12, and 24 h. A fixed amount (2 attomole) of the competitor was added to each sample cDNA and then co-amplified and examined by electrophoresis (2% agarose). The amplified product of competitor is 151 bp shorter than the StAR sequence. M, one-kilobase DNA mol wt marker. Lower panel, graphic representation of the mean expression levels of StAR mRNA in Y-1 cells treated as above. Results are the mean ± SEM of three separate experiments. Media were collected and the progesterone released into the medium was measured by RIA. Results are the mean ± SEM of nine separate experiments. Within each experiment, there were two replicates per treatment. a, Significantly different from the corresponding time point of the control group, $P < 0.05$; b, significantly different from the corresponding time point of the 8-br-cAMP group, $P < 0.05$

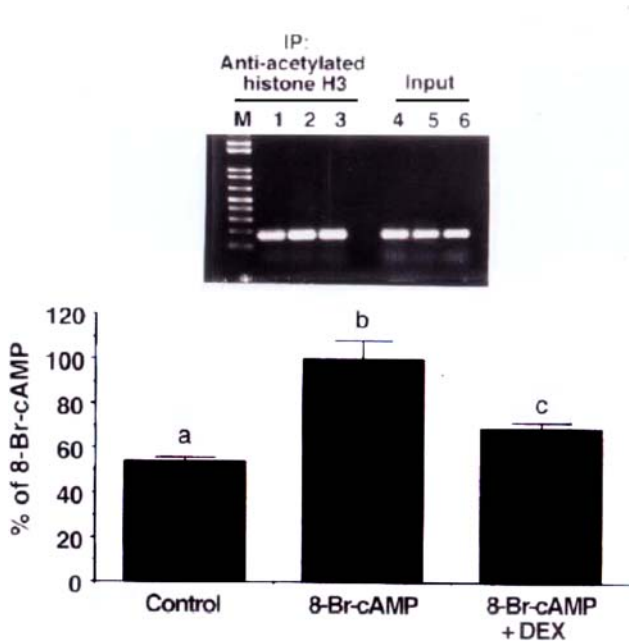

Fig. 5 Chromatin immunoprecipitation analysis of acetylated histone H3 at the StAR promoter after dexamethasone (DEX) treatment of Y-1 cells. Cells were treated with or without (control) DEX (10 μg/ml) in the presence or absence (control) of 8-Br-cAMP (0.5 mM) for 24 h. Soluble chromatin was prepared and immunoprecipitated with anti-acetylated histone H3 antibody. Primers to the StAR promoter were used to amplify the DNA from the same immunoprecipitates. Input DNA is shown as a control for equal amounts of DNA in the samples. Lanes 1 and 4, control; lines 2 and 5, 8-Br-cAMP; lanes 3 and 6, 8-Br-cAMP plus DEX. Results are the mean ± SEM of three separate experiments. Within each experiment, there were two replicates per treatment. The signal intensities of the PCR amplified products were quantified by AlphaImager computer software. Integrated optical density values for the immunoprecipitated DNA sample were normalized to the input chromatin sample. The normalized values were expressed as a percentage of the value of 8-Br-cAMP-treated cells. Results are the mean ± SEM of three separate experiments. Within each experiment, there were two replicates per treatment. Upper panel, a representative PCR product is shown

Effect of treatment with dexamethasone on the acetylation of histone H3 associated with the proximal region of the mouse StAR gene promoter

To determine whether the levels of histone H3 acetylation are decreased in response to either 8-Br-cAMP or 8-Br-cAMP plus dexamethasone at the StAR promoter, we used ChIP assays with an antibody specific for the acetylated form of histone H3. Immunoprecipitated DNA was subjected to PCR with primers amplifying an amplicon within the proximal region of the 5′-flanking region of the mouse StAR gene. Because studies have shown that, in some cellular settings, increased acetylated histone H3, a marker of transcriptional activity, can be associated with the proximal StAR promoter [24]. As shown in Fig. 5, there are no differences in the amounts of targeted sequences in inputs across non-treated and treated cells (lanes 4–6). Treatment with 8-Br-cAMP significantly ($P < 0.05$) stimulated acetylation of histone H3 within the proximal StAR promoter and this effect was inhibited 31% by coincubation with dexamethasone ($P < 0.05$).

To determine whether inhibition of 8-Br-cAMP-stimulated histone H3 acetylation is accompanied by lower levels of acetylated histone H3 protein content in cells treated with the combination of 8-Br-cAMP and dexamethasone for 24 h, we analyzed treated Y-1 cell extracts by Western blotting. As shown in Fig. 6, no significant differences were observed between treatments in total cellular acetylated histone H3.

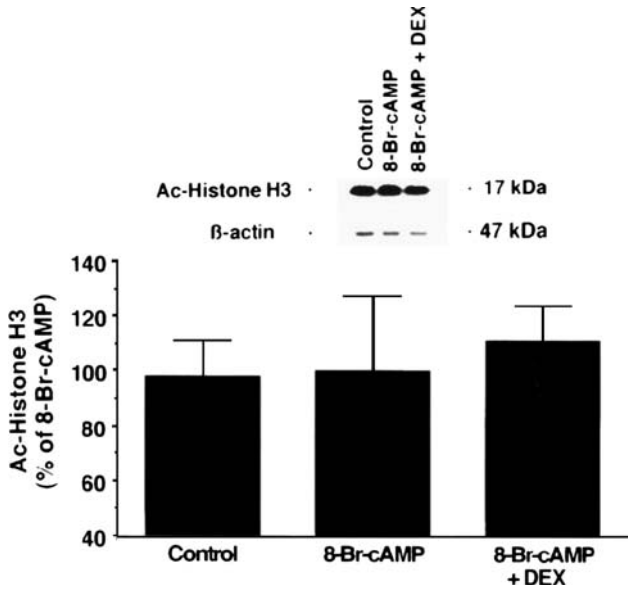

Fig. 6 Effect of dexamethasone (DEX) on the acetylation of histone H3 in Y-1 cells. Cells were treated with or without (control) DEX (10 μg/ml) in the presence or absence (control) of 8-Br-cAMP (0.5 mM) for 24 h. Whole-cell extracts were prepared for Western blot analysis for acetylated (Ac-) histone H3. Blots reblotted with β-actin as a control are shown below the Ac-histone H3 blots. The position of Ac-histone H3 and β-actin is indicated. The Ac-histone H3 and β-actin proteins were detected by chemiluminescence and quantified by AlphaImager computer software. Ac-histone H3 protein values were corrected for the amount of β-actin in each lane. The corrected values were expressed as a percentage of the value of 8-Br-cAMP-treated cells. Results are the mean ± SEM of three separate experiments. Within each experiment, there were two replicates per treatment. Upper panel, a representative Western blot is shown

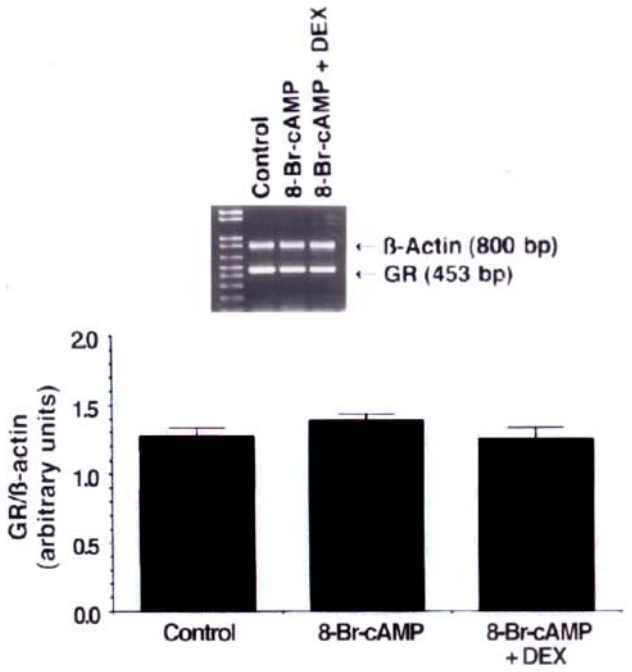

Fig. 7 Effect of dexamethasone (DEX) on glucocorticoid receptor (GR) mRNA expression in Y-1 cells. Cells were treated with or without (control) DEX (10 μg/ml) in the presence or absence (control) of 8-Br-cAMP (0.5 mM) for 24 h. Total RNA was isolated and analyzed by RT-PCR for GR and β-actin mRNAs from three separate experiments. Within each experiment, there were two replicates per treatment. The signal intensities of the PCR amplified products were quantified by AlphaImager computer software. Data were corrected for the amount of β-actin in each lane. Results are the mean ± SEM of GR/β-actin mRNA ratios. Upper panel, a representative PCR product is shown

Effect of treatment with dexamethasone on the expression of GR mRNA

To determine the effects of dexamethasone on GR gene expression, Y-1 cells were treated for 24 h with or without a constant dose of dexamethasone (10 μg/ml) in the presence or absence of 8-Br-cAMP (0.5 mM). RT-PCR analysis revealed that dexamethasone had no effect on GR mRNA expression (Fig. 7).

Discussion

The results of the present study clearly demonstrate the effect of glucocorticoid dexamethasone to inhibit 8-Br-cAMP-induced expression of StAR mRNA and protein in Y-I adrenocortical cells. The dexamethasone effect is dose- and time-dependent and is associated with decreases in progesterone production. These results indicate that dexamethasone inhibits Y-1 cell steroidogenesis by down-regulating StAR mRNA and protein expression. Dexamethasone repression of StAR mRNA accumulation was demonstrated at the level of chromatin, specifically a reduction in 8-Br-cAMP-mediated histone H3 acetylation within the proximal region of the StAR gene promoter. This observation provides a direct mechanism for the down-regulation of StAR gene expression and inhibition of progesterone production.

The studies reported here are in a good agreement with the pervious reports that glucocorticoids, either natural or synthetic, can suppress steroidogenesis at the adrenal gland by diminishing its responsiveness to ACTH in the rat [2, 3, 6, 10], guinea pig [25], domestic fowl [2], and cattle [2, 10]. The inhibitory effect of corticosterone on steroidogenesis of Y-1 adrenal cells has also been reported [9]. These studies confirmed evidence suggesting that the inhibitory action of glucocorticoid on corticosteroidogenesis is at the adrenal level. This observed self-suppression of adrenocortical cells suggests the existence of a mechanism for the adjustment of steroidogenesis that operates in addition to the classical control exerted by the anterior pituitary.

In our studies, the effective inhibitory dose of dexamethasone on StAR mRNA expression was at 10 ng/ml (1.94×10^{-8} M) which is less than circulating levels of

cortisol found in humans under stress, such as hypotension and sepsis, and following trauma or surgery (250 ng/ml) [26–29]. Since, greater than 90% of circulating glucocorticoids are bound by transcortin [30, 31], the dose (10 ng/ml) of dexamethasone, a more potent synthetic glucocorticoid, may represent an overestimate of the amount of free glucocorticoid circulating in the blood of these stressed men, and the effect seen is probably more pharmacological than physiological. Thus, the influence of glucocorticoids in normal adrenal steroidogenesis may be minimal. However, excessive production of glucocorticoids resulting from perturbations of the hypothalamic–pituitary–adrenal axis or the administration of large amounts of glucocorticoid for therapeutic reasons has been shown to alter the function of adrenal glands. Interpretation of corticosteroidogenesis under these conditions may be explained, at least in part, by a direct glucocorticoid action on adrenal cells. A negative feedback loop may exist, thereby providing an additional regulatory pathway modulating the rate of adrenocortical steroid production.

Reports that the GR is present in the adrenal cortex [13] and that the GR antagonist RU486 reverses the inhibitory effect of glucocorticoids on ACTH-induced cortisol production of bovine adrenocortical cells [14] suggest that the effect of glucocorticoid is mediated through the GRs. Several studies have shown that in the majority of cell lines and animal tissues examined, glucocorticoid treatment results in a reduction in GR mRNA [32]. It seems unlikely that the above-mentioned mechanism is effective in our present study. We found that GR mRNA was expressed in Y-1 adrenal cells and that the expression of these receptors was not affected by dexamethasone treatment. The apparent discrepancy might be explained by the differences in experimental animal and (or) conditions used. Although no comparable data are yet available on the synthesis of the GR in Y-1 adrenal cells, it has been shown that dexamethasone at a pharmacologic dose decreased the accumulation of GR mRNA in the adrenal gland in vivo [33]. With two cultured cell types, human IM-9 lymphocytes and rat pancreatic acinar AR42J cells, dexamethasone decreased steady-state GR mRNA levels [34]. Dexamethasone also caused a down-regulation of the levels of GR mRNA in hepatoma tissue culture cells and rat liver in vivo [35]. In contrast to the negative regulative effects of glucocorticoids on GR gene transcription, dexamethasone treatment of glucocorticoid-sensitive leukemia T-cell line, LEM-C7 cells, increased GR mRNA. Our results are in contrast to the down-regulation or up-regulation of GR reported in other cells and tissues. However, our results suggest that regulation of the GR by its cognate ligand may be cell-specific.

In the present study, dexamethasone also decreased 8-Br-cAMP-induced StAR mRNA and protein levels. This suggests that in Y-1 adrenal cells, one inhibitory effect of dexamethasone on steroidogenesis is at the step of StAR. Diminished levels of StAR protein would likely result in a reduction in the rate of adrenocortical cells for progesterone production, in response to ACTH, by limiting the availability of cholesterol to the P450scc complex, located in the inner mitochondrial membrane. Previous studies of the StAR protein have indicated that it has an indispensable role in steroid hormone biosynthesis [17], and it has been further postulated that this role is in cholesterol transfer to the inner mitochondrial membrane [16, 19]. It appears that the dexamethasone-induced depression in progesterone production in Y-1 adrenal cells is due to the inhibition of StAR protein synthesis. This observation is highly consistent with previous studies in which inhibition of steroid hormone biosynthesis has been tightly correlated with StAR synthesis. For example, agents and conditions that have been shown to result in a decrease in steroid hormone biosynthesis, such as helenalin [36], leukemia inhibitory factor [37], lipopolysaccharide [38], PGF2a [39], transforming growth factor-β1 [40], and heat shock [41], have all been demonstrated to decrease StAR protein content. Moreover, a StAR-dependent reduction in steroidogenesis could be manifested through a reduction on the expression and/or activity of the protein. In the present study, dexamethasone treatment reduced StAR mRNA levels. This finding suggests that the inhibitory effect of dexamethasone on StAR occurs as a result of a reduction in cAMP-mediated transcription of the StAR gene and/or StAR mRNA stability. The possibility that dexamethasone may exert a negative effect on StAR activity in adrenal cells through post-translational modification (e.g., phosphorylation) of the protein [42] cannot be ruled out either. Furthermore, StAR gene expression is transcriptionally regulated by the orphan nuclear receptor, steroidogenic factor 1 (SF-1) [43]. The activity of SF-1 is blocked by DAX-1 (dosage-sensitive sex reversal adrenal hypoplasia congenita, critical region on the X chromosome, gene 1) [44]. DAX-1 represses SF-1-mediated transactivation of StAR gene [45]. Therefore, dexamethasone-induced inhibition of StAR may occur via the same mechanism involving SF-1 and DAX-1. Dexamethasone has been shown to increase endogenous DAX-1 expression and concordantly decrease StAR expression in mouse adrenocortical cells [46] and rat testicular cells [47]. This would seem to indicate that up-regulation of DAX-1 gene inhibits StAR gene expression by dexamethasone.

With regard to the direct cellular mechanisms responsible for the suppression of glucocorticoids on StAR gene transcription in adrenocortical cells, we focused our attention on the acetylation of histone H3 on StAR gene

promoter. Association of modified histones with chromatin can determine whether a gene is transcriptionally active or silent [48]. Quantitative ChIP assays are an important tool for analyzing interactions between histones and native chromatin [49]. Three studies have looked at the association of different proteins (including modified histones) with the StAR gene promoter or coding sequence in MA-10 Leydig cells [23], hCG-primed mouse granulosa cells [24], and FSH-stimulated porcine granulosa cells [50]. Results of the present study demonstrate that 8-Br-cAMP stimulation of StAR mRNA accumulation in Y-1 adrenal cells is associated with increased acetylation of histone H3 in the StAR gene promoter. Dexamethasone reduced the ability of 8-Br-cAMP to increase histone H3 acetylation in the proximal region of the endogenous StAR promoter. Acetylation of histone H3 at lysines 9 and 14 is linked to transcriptional activation [48]. The finding that 8-Br-cAMP stimulation of histone H3 associated with the StAR proximal promoter is in agreement with data for the murine gene in MA-10 cells [24]. Hiroi et al. [24] also observed that this modification occurs in MA-10 cells as early as 15 min after 8-Br-cAMP treatment. This rapid modification in MA-10 cells is accompanied by significant increases in StAR mRNA seen within 30 min. Since we detected significant histone H3 acetylation at 24 h of treatment with 8-Br-cAMP, the slower increase in StAR mRNA in Y-1 cells that was significantly increased at 6 h of 8-Br-cAMP treatment leads us to suggest that histone acetylation is minimal at 1 and 3 h of 8-Br-cAMP treatment in our model system. Our result, however, provide additional evidence for the correlation between histone H3 acetylation and StAR gene activation. Previous studies have indicated that histone acetylation and deacetylation are governed by the opposing activities of two enzyme classes: histone acetyltransferase (HAT) and histone deacetylases (HDAC) [51, 52]. The overall acetylation status of histones depends on the dynamic equilibrium between HAT and HDAC activities. Induction of HDAC activity can lead to reduced histone acetylation and decreased gene expression [49, 53]. Thus, the dexamethasone-induced repression of histone H3 acetylation at the StAR promoter may be due to an increase in HDAC activity resulting in enhanced nucleosome compaction and consequent repression of StAR gene transcription. The activation of nucleosomal histone by HAT such as p300 and CREB (cAMP response element binding protein)-binding protein (CBP) causes local unwinding of DNA and stimulates transcription [54]. Studies in A549 cells showed that dexamethasone inhibited CBP-associated HAT activity [55]. Thus, HAT may be another potential target of dexamethasone action in the regulation of StAR gene transcription. Whether dexamethasone inhibits 8-Br-cAMP-stimulated histone acetylases or dexamethasone stimulates histone deacetylases will require further study. In addition, dexamethasone may activate DNA methyltransferase resulting in hypermethylation and silencing of StAR gene. Due to gonadotropin-induced activation of the StAR gene did reduce dimethylation of lysine 9 of histone H3 [24].

Our Western blotting data demonstrated that 8-Br-cAMP and 8-Br-cAMP plus dexamethasone did not promote a detectable change in bulk levels of acetylated histone H3 compared with control. Studies in rat and pig granulosa cells reported that FSH did not alter the levels of acetylated histone H3 when compared with control [50, 56]. These studies support the idea that total cellular levels of histone H3 acetylation are fairly constant and increases in histone H3 acetylation are restricted to target genes.

In conclusion, the results of the present study indicate that in mouse Y-1 adrenocortical cells, dexamethasone acts to repress the 8-Br-cAMP-induced expression of StAR and production of progesterone. Dexamethasone also suppresses histone H3 acetylation at the StAR promoter. These observations raise the possibility that glucocorticoids in vivo may act directly on the corticosteroid-producing cells of the adrenal gland. This may contribute to the adrenal suppression seen with therapeutic glucocorticoid administration.

Materials and methods

Materials

Bovine serum albumin (BSA; fraction V, fatty-acid free), 8-bromo-adenosine 3′,5′-cyclic monophosphate (8-Br-cAMP), proteinase K, salmon sperm DNA, and other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO), unless otherwise stated. Dexamethasone (dexamethasone phosphate) was obtained from Narn Guang Chemical Co. (Tainan, Taiwan). Ham's F12/Dulbecco's modified Eagle's medium (F12/DMEM; 1:1), fetal calf serum (FCS), and other culture supplies were purchased from Gibco-BRL (Grand Island, NY). [a-^{32}P] Deoxy-CTP (3,000 Ci/mmol) was obtained from NEN Life Science Products (Boston, MA). Rabbit anti-acetylated histone H3 antibody was purchased from Upstate Biotechnology (Lake Placid, NY). Horseradish peroxidase-conjugated donkey anti-rabbit IgG antibody was purchased from Amersham Life Science (Buckinghamshire, England). Mouse StAR cDNA and StAR antiserum were provided by Dr. D.M. Stocco (Department of Cell Biology & Biochemistry, Texas Tech University Health Sciences Center, Lubbock, TX). Mouse glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA was a generous gift from Dr. Hsiao-Sheng Liu (Department of Microbiology, College of Medicine, National Cheng Kung University, Tainan, Taiwan).

Cell culture

The mouse Y-1 adrenocortical tumor cells, a generous gift from Dr. Bernard P. Schimmer (Banting and Best Department of Medical Research, University of Toronto, Toronto, Ontaria, Canada) were maintained in F12/DMEM supplemented with 20 mM HEPES (pH 7.4), 400 mM glutamine, 100 IU/ml penicillin, and 100 μg/ml streptomycin in the presence of 10% FCS. Cells were grown in 75-cm^2 tissue culture flasks at 37°C in a 5% CO_2-air atmosphere and subcultured into 100-mm tissue culture dishes at a density of approximately 2.5×10^6 cells 24 h prior to use in experiments. The Y-1 cells have a reduced capacity for 21-hydroxylation and 11-hydroxylation reactions compared with primary adrenal cells. They synthesize progesterone and accumulate P450scc mRNA after stimulation of 8-Br-cAMP [57].

Mitochondrial protein and whole-cell extract preparation

Mitochondrial protein preparations were performed as previously described [58] with slight modifications. After addition of 1.0 ml of TSE buffer (10 mM Tris–HCl, 250 mM sucrose, 0.1 mM EDTA, pH 7.4), the cells were scraped off the dish with a rubber policeman and sonicated (50% amplitude, 10 s/cycle, 3 cycles; Dr. Hielschel Sonicator, Model: UP-50 H; Senecoscience Prestinar, Milano, Germany). After centrifugation at 600*g* for 30 min at 4°C, the supernatant was collected and centrifuged at 13,800*g* for 30 min at 4°C. The pellet was resuspended in 300 μl of TSE buffer. Whole-cell lysates were prepared by lysing Y-1 cells in 0.3 ml of RIPA lysis buffer (10 mM Tris–HCl, 150 mM NaCl, 5 mM EDTA, 0.1% sodium dodecyl sulfate (SDS), 1% Nonidet P-40 (NP-40), 0.25% deoxycholate, 1 mM sodium vanadate, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 μg/ml leupeptin, and 1 μg/ml aprotinin). The cells were scraped off the dish with a rubber policeman and rotated for 20 min at 4°C. Cells were then passed through a 23-gauge needle to shear the DNA. After centrifugation at 13,800*g* for 10 min at 4°C, the supernatant was removed. Protein concentrations in the mitochondrial protein and whole-cell lysate extracts were determined by Lowry's method [59]. All samples were stored at −85°C until further analysis.

Western blot analysis

Fifty micrograms of whole-cell lysates and mitochondrial protein were analyzed as previously described [58]. The protein samples were separated by 12.5% SDS-PAGE, transferred to polyvinylidene difluoride membrane, and then the membranes were blocked in phosphate-buffered saline (PBS) containing 0.5% Tween 20 (PBST) and 4% non-fat dry milk for 1 h. The membrane was incubated with primary antibody (anti-StAR, 1:2,000 or anti-acetylated histone H3, 1:400) in PBST with 0.1% BSA and immunoblot analysis performed using a horseradish peroxidase-conjugated donkey anti-rabbit IgG antibody (1:3,000). Specific immunoactive bands were detected using the Western lightening Chemiluminescent Kit (NEN Life Science Products). Band intensities were determined using either the Arcus II computer-assisted image system (PDI Inc., Huntington Station, NY) or AlphaImager 2200 system (AlphaInnotech Inc., San Leandro, CA). Values obtained were expressed as integrated optical density units.

Northern blot analysis

Levels of StAR mRNA in the dose–response effect of dexamethasone were evaluated by Northern blot analysis using a mouse StAR cDNA probe as previously described [60]. Total RNA (25 μg) from each sample was fractionated in a 1% agarose gel and transferred onto a nylon membrane and UV cross-linked. Northern blots were pre-incubated for 1 h at 60°C in QuikHyb solution (Stratagene, La Jolla, CA) and hybridized for 3 h at 60°C in the same solution containing heat-denatured [^{32}P]-labeled StAR or GAPDH cDNA and salmon sperm DNA. Mouse StAR and GAPDH probes were labeled by random priming with [α-^{32}P]deoxy-CTP using the Megaprime DNA Labeling System (Amersham). After hybridization, the blots were washed twice in 2× SSC/0.1% SDS at room temperature for 15 min each, and once in 0.1× SSC/0.1% SDS for 20 min at 42°C. After washing, the membrane was exposed to X-ray film at −85°C for 24 h for autoradiographic signals. The intensity of the bands on Northern blots was measured by the Arcus II computer-assisted image system. Values for StAR mRNA were normalized to values for the GAPDH.

Construction of the native and competitor templates for StAR

To quantitate the mRNA level of StAR in Y-1 cells treated with or without dexamethasone (10 μg/ml) in the presence of 8-Br-cAMP (0.5 mM) for various time intervals, we adopted standard curve quantitative, competitive (QC)-reverse transcription (RT)-polymerase chain reaction (PCR; QC-RT-PCR) method as described previously [61] using a competitive internal standard that consisted of a shortened StAR DNA fragment as illustrated in Fig. 3a.

Specific primer pairs for StAR designed according to sequences deposited in GenBank No. L36063 were as follows: sense 5′-AGATGTGGGCAAGTGTGTTTC-3′ (P1), antisense 5′-CAGGTCAATGTGGACAG-3′ (P2), and internal reverse primer 5′-CAGGTCAATGTG GTGGACAGTGAGCAGCCAAGTGAGTTTAG-3′ (IP). The IP, a 41 base-pair (bp) primer, was designed to include a sequence of 21 bp (underlined, matching the solid-lined region in Fig. 3a) that was 171 bp upstream from the P2 sequence appended to the 3′-end of P2 sequence. Thus, using P1 as the 5′-primer and P2 as the 3′-primer, a 540 bp of the StAR sequence was amplified. With the appended 21 bp of primer IP as the 3′-primer, this resulted in a 389 bp (369 bp + 20 bp from P2) amplicon. An internal region of 151 bp was deleted from the 540-bp DNA fragment.

QC-RT-PCR

Mouse StAR mRNA isolated from Y-1 cells was reverse-transcribed using oligo(dT)$_{15}$ (MWG Biotech, Taipei, Taiwan) and SuperScript II M-MLV RNase H$^-$ reverse transcriptase (Invitrogen, Carisbad, CA) according to manufacturer's instruction. Two microliter of RT product was PCR-amplified using a forward (P1) and a reverse (P2) primer. A 540-bp PCR fragment was obtained and cloned into pCRII vector (Invitrogen), which represented plasmid containing "native" sequence. Using a forward (P1) and an internal reverse (IP) primer, a DNA fragment of 389 bp was amplified by PCR and then subcloned into pCRII vector, representing plasmid containing "competitor" sequence. Both plasmids were linearized by *Kpn*I restriction enzyme (Roche Molecular Biochemicals, Mannheim, Germany) and transcribed in vitro using T7 RNA Polymerase (Promega, Madison, WI). RNase-free DNase I (Sigma) was used to remove plasmid DNA from RNA pool. In vitro-transcribed RNA was precipitated twice with 1/10 volume of 2 M sodium acetate (pH 4.0) and two volumes of 100% ethanol after phenol–chloroform extraction. The concentration of RNA was determined by 260 nm absorbance. RNA was then aliquoted and stored at −85°C until used.

A constant amount (1.5 fentomole) of competitor and native RNA in 2 μl of diethylpyrocarbonate-treated water or 0.1 μg of unknown mRNA samples was added into 0.2-ml thin-wall PCR tubes (ABgene, Rochester, NY) containing 12 μl of 1× RT master mixture [1× RT buffer, 10 mM dithiothreitol (DTT), 0.5 μg oligo(dT)$_{15}$, 2 mM deoxynucleoside triphosphate (dNTPs), and 50 U SuperScript RNase H$^-$ reverse transcriptase (Invitrogen)]. The final volume of RT mixture was 30 μl, and reverse transcription was performed at 42°C for 75 min followed by heating to 95°C for 10 min and quick-chilled to 4°C in a programmable thermal cycler (ABI model 2700, Perkin-Elmer Corp., Foster City, CA). RT products of unknown samples were diluted 1:100 with double-distilled water, while RT products of native and competitor RNA were diluted to 25.6 attomole and 2 attomole per 2 μl double-distilled water, respectively. The standard QC-RT-PCR assay contained 2 attomole competitor RNA and eight serial dilutions of native RNA (from 25.6 to 0.2 attomole). Two microliter of the diluted competitor RT products was added into 7 μl of 1× PCR mixture [1× PCR buffer, 0.2 mM dNTPs, 0.5 U *Taq* DNA Polymerase (ABgene), and 0.4 μM of primers]. This mixture was then dispensed into 0.2-ml thin-wall PCR tubes, and 2 μl of the diluted native RT products (serially diluted) or 2 μl of unknown sample RT products were added individually to each tube. The final volume of PCR mixture was 25 μl. The PCR mixture was heated for 4 min at 95°C as the initial denaturation. PCR amplifications were then performed under the following conditions: 30 s denaturation at 94°C, 30 s annealing at 65°C, and 40 s elongation at 72°C for 35 cycles, followed by 5 min at 72°C as the last primer extension. Ten microliter of PCR products were directly separated on 2% agarose gel with 1× TAE buffer (45 mM Tris–acetate, 1 mM EDTA, pH 8.0) at 100 V for 30 min using Mupid-2 Mini-Gel Electrophoresis Unit (Cosmo Bio Co. Ltd., Tokyo, Japan). The gel was then stained with ethidium bromide and photographed under UV illuminator equipped with a camera connected to a computer (Fig. 3b). The gel image was analyzed using AlphaImager software. A ratio was calculated for the intensity of native versus competitor bands on each lane of the gels. The logarithmic ratio of native to competitor was plotted against the logarithmic amount of initial native to produce the standard curve (Fig. 3c), and concentrations of specific mRNA transcripts were determined by comparison to the standard curve.

Chromatin immunoprecipitation (ChIP) assay

A modification of the technique described by Li et al. [62] for ChIP assay was used. Formaldehyde (Sigma) was added directly to the cell culture medium at a final concentration of 1% for 15 min (37°C) to cross-link DNA and its associated proteins. Cross-linking was terminated upon addition of glycine (125 mM final concentration) for 10 min. Cells were washed once in PBS before scraping in PBS containing protease inhibitors (1 mM PMSF, 1 μg/ml aprotinin, and 1 μg/ml leupeptin). Cells were resuspended in 400 μl SDS lysis buffer (50 mM Tris–HCl, pH 8.1, 1% SDS, 10 mM EDTA) containing protease inhibitors, followed by incubation on ice for 10 min. Samples were then

sonicated on ice for three times for 30 s each. Sonicated samples were centrifuged (13,800*g*, 10 min, 4°C) to spin down cell debris. The chromatin solution was diluted with 1.1 ml of dilution buffer (16.7 mM Tris–HCl, pH 8.1, 1.2 mM EDTA, pH 8.1, 0.01% SDS, 1.1% Triton X-100, 1 mM PMSF, 1 μg/ml aprotinin, and 1 μg/ml leupeptin). A supernatant fraction (50 μl) from diluted chromatin mixtures was saved as an input control. The chromatin preparation was precleared with 80 μl of salmon sperm DNA-protein A/Sepharose (Amersham; 20% slurry) for 30 min at 4°C with rotation. One part of the chromatin complexes was then incubated with 5 μg of anti-acetylated histone H3 antibody and rotated at 4°C overnight. The other was incubated with normal rabbit serum as non-immune serum control. Immune complexes were collected with 60 μl of salmon sperm DNA-protein A/Sepharose (20% slurry) with rotation for 1 h at 4°C. The supernatant was collected as an unbound control. Immunoprecipitates were sequentially washed for 5 min in wash buffer A (20 mM Tris–HCl, pH 8.1, 2 mM EDTA, 0.1% SDS, 1% Triton X-100, 150 mM NaCl), wash buffer B (2 mM Tris–HCl, pH 8.1, 2 mM EDTA, 0.1% SDS, 1% Triton X-100, 500 mM NaCl), wash buffer C (10 mM Tris–HCl, pH 8.1, 1 mM EDTA, 250 mM NaCl, 1% NP-40, 1% sodium deoxycholate), and TE buffer (10 mM Tris–HCl, 0.1 mM EDTA, pH 8.0) (two times). Washed beads were extracted with 250 μl of elution buffer (1% SDS, 100 mM $NaHCO_3$) two times. The elutes were combined in one tube, treated successively for 4 h at 65°C in 200 mM NaCl (20 μl) to reverse cross-links and then incubated for 40 min at 57°C with 40 μg/ml of proteinase K in proteinase K buffer (40 mM Tris–HCl, pH 6.5, 10 mM EDTA). The DNA was extracted with phenol/chloroform, ethanol precipitated, and diluted in 10 μl double-distilled water. StAR promoter associated DNA was amplified by PCR. Each PCR mixture contained 2 μl of immunoprecipitate, unbound or input control, 0.4 μM each primer, 0.2 mM dNTPs mixture, 2.5 units of *Thermus icelandicus Taq* DNA polymerase (ABgene) and 1× *Taq* PCR buffer in a total volume of 25 μl. The primers for the mouse StAR promoter were selected to include the proximal promoter when a large number of the described transcriptional response elements reside (−68 to −153). The primers are as follows: forward, 5′-ACCTGCAGAGTCTGGTCCTC-3′ (bases −153 to −135); reverse, 5′-TCAAGTGCGCTGCCTTAAAT-3′ (bases −88 to −110). PCR amplifications were carried out under the following conditions: 30 s at 94°C, 30 s at 60°C, and 40 s at 72°C for 35 cycles, preceded by 4 min at 95°C and followed by 5 min at 72°C. The PCR products were separated in 2% agarose gels. The gel was stained with ethidium bromide and then placed on a UV illuminator equipped with a camera connected to a computer. The gel image was analyzed using AlphaImager software.

RT-PCR

To determine the mRNA level of GR in Y-1 cells treated with or without dexamethasone in the presence or absence of 8-Br-cAMP for 24 h, total RNA was analyzed by RT-PCR. One hundred nanogram of RNA dissolved in sterilized water was used for the RT. First strand synthesis was performed in 30 μl of 5× RT buffer, 10 mM DTT, 0.5 μg $oligo(dT)_{15}$, 0.2 mM dNTPs, and 6.67 units of murine leukemia virus reverse transcriptase (ABgene), and 0.1 μg of total RNA. The reaction was incubated at room temperature for 10 min, at 42°C for 75 min, at 95°C for 10 min, and then at 4°C in a GeneAmp 2700 PCR system. The primers for the mouse GR are as follows: forward, 5′-ACCTCGATGACCAAATGACC-3′; reverse, 5′-TCTGGAAGCAGTAGGTAAGGAGA-3′. Forward and reverse primers for mouse β-actin are as follows: forward, 5′-ATCTGGCACCACACCTTCTACAATGAGCTGCG-3′; reverse, 5′-CGTCATACTCCTGCTTGCTGATCCACATCTGC-3′. PCR reactions were performed on 2 μl of the cDNA in a final reaction volume of 25 μl in the presence of 2.5 units of *Thermus icelandicus Taq* DNA polymerase (ABgene). After denaturation at 95°C for 4 min, PCR amplification was carried out by 35 cycles of incubation at 94°C for 30 s, 58°C for 30 s, and 72°C for 40 s, followed by a final extension at 72°C for 5 min. The PCR products (10 μl) were electrophoresed in a 2% agarose gel with 1× TAE buffer (45 mM Tris–acetate, 1 mM EDTA, pH 8.0) at 100 V for 30 min using Mupid-2 Mini-Gel Electrophoresis Unit. The gel was then stained with ethidium bromide and placed on a UV illuminator equipped with a camera connected to a computer. The signal intensities of the PCR amplified products were quantified using AlphaImager computer software.

Radioimmunoassay (RIA) for progesterone

Quantitation of progesterone directly from aliquots of the medium was performed by RIA as previously described [63]. The antiserum to progesterone-11α-BSA (C467-B4) was supplied by Dr. J.E. Hixon (Department of Veterinary Biosciences, College of Veterinary Medicine, University of Illinois, Urbana, IL), and used at the dilution of 1:25,000 in 0.1 M Tris buffer (pH 7.4). The intra-assay variation, determined by duplicates of three dose levels of the control medium from cultured pig corpora luteal cells, was less than 10%. The sensitivity of the assay was 12.5 pg per assay tube.

Statistical analysis

Two means were compared using Student's *t*-test. Where there were more than two means, significant differences

between means were determined by analysis of variance (ANOVA). The means were then analyzed by Fisher's PLSD multiple comparison.

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