Glucocorticoids antagonize cAMP-induced Star transcription in Leydig cells through the orphan nuclear receptor NR4A1

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Abstract

It is well established that stress, either physical or psychosocial, causes a decrease in testosterone production by Leydig cells. Glucocorticoids (Gc) are the main mediators of stress response and they convey their repressive effect on Leydig cells through the glucocorticoid receptor (GR). So far, various mechanisms have been proposed to explain the mechanism of action of Gc on Leydig cell steroidogenesis including repression of genes involved in testosterone biosynthesis. Several steroidogenic genes, including steroidogenic acute regulatory (STAR) protein, have been shown to be repressed by Gc in a GR-dependent manner but the underlying mechanisms remain to be fully elucidated. Here, we found that dexamethasone (Dex), a potent synthetic Gc, partly antagonizes the cAMP-dependent stimulation of the mouse Star promoter in MA-10 Leydig cells as revealed by transient transfection assays. This repression requires an element located at −95 bp previously implicated in the activation of the Star promoter by the nuclear receptors, NR4A1 and NR5A1. Dex was found to inhibit NR4A1-dependent transactivation of the Star promoter in Leydig cells by decreasing NR4A1, but not NR5A1, recruitment to the proximal Star promoter as determined by chromatin immunoprecipitation assay. Western blots revealed that Dex did not affect NR4A1 or NR5A1 expression in response to cAMP. These data suggest that NR4A1 would be associated with the GR in a transcriptionally inactive complex as previously demonstrated in pituitary corticotrope cells. Thus, our data provide new molecular insights into the stress-mediated suppression of testosterone production in testicular Leydig cells.

Introduction

It is well recognized that elevated glucocorticoid (Gc) levels resulting from diverse stressful conditions lead to the suppression of serum testosterone (Hardy et al. 2005). Gc-mediated suppression of testosterone has profound physiological consequences, including muscular atrophy and sexual dysfunction (Charpentier et al. 1981, Cumming et al. 1983, Rabin et al. 1988, Contreras et al. 1996). In humans, elevated cortisol, the main circulating Gc, resulting from insulin-induced hypoglycaemia or Cushing’s syndrome due to adrenal hyperplasia or adenoma, decreases serum testosterone content, with no alteration in pituitary luteinizing hormone (LH) levels (Gabrilove et al. 1974, Smals et al. 1977, Cumming et al. 1983, Vierhapper et al. 2000, Stikkelbroeck et al. 2001). A similar pattern is also observed in rats subjected to immobilization stress (Orr & Mann 1990, 1992, Orr et al. 1994, Dong et al. 2004) and psychosocial stress (Hardy et al. 2002). Further supporting the repressive role of Gc on testosterone production is the fact that the reduction of endogenous corticosterone levels (the main Gc in rodents) leads to increased testosterone production by Leydig cells (Gao et al. 1996).

Leydig cells express the Gc receptor (GR, NR3C1) and are the main targets of Gc action in the testis (Evain et al. 1976, Stalker et al. 1989, 1991, Schwarzenbach et al. 2003). Although the molecular mechanisms by which Gc decrease steroidogenesis have not been entirely defined, numerous studies have shown that Gc can inhibit expression of enzymes involved in testosterone biosynthesis such as Star, Cyp11a1, Cyp17a1, Hsd3b and Hsd17b3 (Hales & Payne 1989, Payne & Sha 1991, Wang et al. 2000, Huang & Shirley 2001, Schwarzenbach et al. 2003, Badrinarayanan et al. 2006, Fon & Li 2007). Interestingly, variations in serum Gc content have no significant effect on circulating levels of LH and on its binding properties to its receptor, indicating that Gc repressive effects on Leydig cell steroidogenic capacity are not mediated by an indirect effect on pituitary LH production (Orr & Mann 1990, Orr et al. 1994, Gao et al. 1996). In addition to repressing expression of steroidogenic enzyme-encoding genes, Gc were also reported to induce Leydig cell apoptosis (Gao et al. 2002, 2003, Wang & Gao 2006).

The initiation of steroid biosynthesis requires the transport of cholesterol from the outer to the inner mitochondrial membrane. This is mediated by a protein...
complex that comprises the steroidogenic acute regulatory (STAR) protein (reviewed in Manna & Stocco 2005). Mutations in the Star gene in humans (naturally occurring) and in mice (by genetic manipulations) cause male pseudhermaphroditism and lipid congenital adrenal hyperplasia accompanied by a loss in steroid synthesis in the gonads and adrenal (reviewed in Stocco 2002). In Leydig cells, Star gene expression, and consequently steroidogenesis, is mainly regulated by LH-mediated activation of cAMP-dependent pathways ultimately leading to transcriptional activation (Ascoli et al. 2002). Several transcription factors including NR5A1 (SF1), GATA4, CEBPB, SREBF1, SP1, CREB1/CREM, FOS/JUN, and NR4A1 have been shown to activate Star promoter activity while NR0B1 (DAX1) represses it (reviewed in Stocco et al. 2001, Manna et al. 2003, Manna & Stocco 2005 and Ref. Martin et al. 2008). Of all these factors, the orphan nuclear receptor NR4A1 was shown to be rapidly and strongly induced in response to LH/cAMP in Leydig cells (Song et al. 2001, Martin & Tremblay 2005, Martin et al. 2008).


Gc are known to directly modulate testosterone biosynthesis in Leydig cells through GR-mediated repression of genes encoding various components of the steroidogenic pathway. Gc-activated GR normally binds as a homodimer to a consensus DNA sequence GGTACAnnnTGTTCT called the Gc response element (GRE) found in the promoter region of target genes resulting in either transcriptional activation or repression (Schoneveld et al. 2004). The promoters of steroidogenic genes repressed by Gc, however, do not contain GREs, which strongly suggests an indirect mechanism. Here, we report that the repression of mouse Star promoter activity in Leydig cells by dexamethasone (Dex), a GR agonist, involves the nuclear receptor NR4A1. Dex-mediated repression of NR4A1- and cAMP-dependent activation of Star promoter activity was lost when a mutation was introduced in the NR4A1-responsive element at −95 bp. Finally, we show that Dex inhibits the recruitment of NR4A1, but not NR5A1, to the Star promoter.

Materials and methods

Chemicals

Dex and 8-Br-cAMP were purchased from Sigma–Aldrich Canada.

Plasmids

The −902 and −71 bp murine Star luciferase reporter constructs have been described previously (Tremblay & Viger 2001). The −902 bp Star reporter construct harbouring a mutation inactivating the NBRE/NR5A1 element at −95 bp (CATCCCCGA to CATAATTGA) has been described previously (Martin et al. 2008). Expression vectors for NR4A1 and GR (Philips et al. 1997a) were provided by Dr Jacques Drouin (Laboratoire de Génétique Moléculaire, Institut de Recherches Cliniques de Montréal, Montréal, Canada).

Cell culture and transfections

Mouse MA-10 Leydig cells (Ascoli 1981) were provided by Dr Mario Ascoli (University of Iowa, Iowa City, IA, USA). MA-10 Leydig cells were grown in Waymouth’s MB752/1 medium supplemented with 1·2 g/l NaHCO3, 15% horse serum and 50 mg/l gentamycin and streptomycin sulphate, at 37 °C in 5% CO2 and transfected as described previously (Martin et al. 2008). In experiments involving hormonal treatment, cells were treated with 0·5 mM 8-Br-cAMP for 4 h prior to harvesting in the absence or the presence of Dex (100 nM) that was added 30 min before cAMP. Data reported represent the average of at least three experiments, each performed in duplicate.

Protein purification and western blots

MA-10 Leydig cells were incubated in serum-free medium and treated with either vehicle or Dex (100 nM) for 30 min before addition of 0·5 mM 8-Br-cAMP for 2 or 4 h. MA-10 Leydig cells were then rinsed twice with ice cold PBS and nuclear proteins were isolated according to the procedure outlined
by Schreiber et al. (1989). Protein concentrations were estimated using standard Bradford assay. In a denaturing loading buffer, 20 μg aliquots of nuclear proteins were boiled for 10 min, fractionated by SDS-PAGE and transferred onto Polyvinylidene Fluoride membrane (Millipore, Bedford, USA). Immunodetection was performed using an avidin–biotin approach according to the manufacturer’s instructions (Vector Laboratories Inc., Ontario, Canada). The detection of NR4A1, NR5A1, LMNB1 (which serves as a loading control) was performed using a monoclonal anti-NR4A1 antibody (1:500 dilution; BD Biosciences Pharmingen, San Diego, CA, USA), a polyclonal anti-NR5A1 antibody (1:2000 dilution; ABR-Affinity BioReagents, Golden, CO, USA) and a polyclonal anti-LMNB1 antibody (1:200 dilution; Santa Cruz Biotechnologies, Santa Cruz, USA).

RNA isolation, reverse transcription and quantitative real-time PCR

Total RNA was isolated from MA-10 Leydig cells using RNeasy Plus extraction kit (Qiagen Inc). First-strand cDNAs were synthesized from a 1 μg aliquot of the various RNAs using the Transcripter Reverse Transcriptase System (Roche Diagnostics). MA-10 Leydig cells were grown in serum-free medium containing either vehicle or 100 nM Dex for 30 min before addition of 0.5 mM 8-Br-cAMP for the indicated time prior to RNA isolation. Quantitative real-time PCR was performed using a LightCycler 1.5 instrument and the LightCycler FastStart DNA Master SYBR Green I kit (Roche Diagnostics) according to the manufacturer’s protocol. 

Statistical analyses

To identify significant differences within multiple groups, statistical analyses were done using one-way ANOVA followed by Bonferroni’s multiple comparison test. Comparisons between two groups were done using Mann–Whitney U test. For all statistical analyses, \( P<0.05 \) was considered significant. All statistical analyses were done using the SigmaStat software package (Systat Software Inc., San Jose, CA, USA).

Results

Gc repress cAMP-mediated induction of Star expression in Leydig cells

We first quantified by real-time PCR the effect of Dex, a potent synthetic Gc, on Star expression in response to cAMP stimulation in a time-dependent manner in MA-10 Leydig cells. As expected, Star mRNA levels were robustly increased in response to cAMP in a time-dependent manner (Fig. 1, open bars). In the presence of Dex, however, the cAMP-mediated induction of Star mRNA was significantly repressed by 35% at 4 and 6 h (Fig. 1, solid bars), while the reduction observed at 2 h was not statistically significant (\( P=0.08 \)).

Gc antagonize NR4A1- and cAMP-dependent activation of the mouse Star promoter

Since Gc can repress gene expression by antagonizing NR4A1-dependent transactivation in the pituitary (Philips et al. 1997b, Martens et al. 2005), and since we have found that NR4A1 can activate the mouse Star promoter (Martin et al. 2008), we tested whether Dex could inhibit NR4A1 action on the mouse Star promoter in the MA-10 Leydig cells. The treatment of

Chromatin immunoprecipitation (ChIP) assay

ChIP assays were performed as described previously (Robert et al. 2006). The genomic DNA immunoprecipitated with the NR4A1 or NR5A1 antisera was directly analysed by quantitative real-time PCR using primers specific for the proximal region (−299 bp to −41 bp) of the mouse Star promoter (forward: 5′-TGA TGC ACC TCA GTT GGT GG-3′ and reverse 5′-GCT GTG CAT CAT CAC TTG AG-3′). The conditions were as follows: 10 min at 95 °C followed by 35 cycles of denaturation (5 s at 95 °C), annealing (5 s at 62 °C) and extension (20 s at 72 °C) with single acquisition of fluorescence at the end of each extension step. Absolute quantification of Star promoter DNA fragments immunoprecipitated with the NR4A1 or NR5A1 antisera was performed using a standard curve generated with known concentrations of the −902 bp Star promoter construct and is expressed as a ratio of Star promoter immunoprecipitated DNA to input DNA levels. Input DNA represents 5% of total DNA used for a ChIP experiment.

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MA-10 Leydig cells with Dex alone in the absence of any stimulation had no effect on the activity of a −902 bp mouse Star reporter construct (Fig. 2A). NR4A1 transactivated the Star reporter construct by about threefold and this activation was significantly repressed by 21% by Dex in MA-10 Leydig cells (Fig. 2A). Similarly, the treatment of MA-10 Leydig cells with 8-Br-cAMP led to a fivefold activation of the Star promoter that was repressed by 38% by Dex (Fig. 2B). Combination of NR4A1 and cAMP, which further enhances Star promoter activation, was also repressed by Dex (Fig. 2B). Because Gc repressive effects are known to be mediated through GR (Bambino & Hsueh 1981, Huang & Shirley 2001), we speculated that the overexpression of this receptor might enhance the effect of Dex. We therefore performed the same experiments in the presence of an expression vector encoding GR. We found that Dex-mediated repression of NR4A1- (Fig. 3A) and cAMP-stimulated (Fig. 3B) Star promoter activity was more robust in the presence of GR and reached 50% when both NR4A1 and cAMP were combined (Fig. 3B). These results further support the implication of GR in the Dex-mediated repression of Star promoter activity. The inhibitory effect of Dex on the NR4A1- and cAMP-mediated activation of Star transcription was found to be specific since it was lost when a minimal Star reporter construct (−71 bp) was used (Fig. 4). The weak but significant NR4A1- and cAMP-dependent activation of the −71 bp Star promoter construct may be attributed to the presence of other regulatory elements including a NR5A1 binding element at −45 bp (Fig. 4).

**Dex-mediated repression requires an intact element at −95 bp**

We have recently found that NR4A1-dependent activation of the Star promoter requires an intact NRE/NR5A1 element at −95 bp (Martin et al. 2008). We therefore tested whether this element might be involved in the repression by Dex/GR of the NR4A1/cAMP-stimulated mouse Star promoter in Leydig cells. As shown in Fig. 5A, a −902 bp mouse Star promoter construct harbouring a two nucleotide mutation in the −95 bp NRE/NR5A1 element was no longer activated by NR4A1 and the repressive effect by Dex/GR was lost. Although this mutated Star construct could still be activated by cAMP—most likely through other regulatory elements for transcription factors such as NR5A1, GATA4, CEBPB and AP-1 known to be
involved in cAMP-dependent regulation of the Star transcription (reviewed in Manna et al. 2003) – the repression by Dex/GR was completely abolished (Fig. 5B). In terms of repression by Dex, the -902 bp reporter construct that contains a mutation in the NBRE/NR5A1 element at K95 bp behaved exactly as the minimal K71 bp Star reporter (Fig. 4). Altogether, these results indicate that an intact NBRE/NR5A1 element at K95 bp is essential for the Dex/GR-mediated repression of the NR4A1/cAMP-induced Star promoter activity.

Dex decreases NR4A1 recruitment to the proximal Star promoter in response to cAMP

Because the −95 bp element, which we found to be essential for the Dex repressive effects on the Star
promoter (Fig. 5), is important for NR4A1- and NR5A1-mediated transactivation of the Star promoter, we hypothesized that Dex/GR might target these transcription factors. We first tested whether the repression by Dex/GR could be attributed to a decrease in NR4A1 and NR5A1 protein levels. As shown in Fig. 6, western blots revealed that Dex had no significant effect on the cAMP-induced NR4A1 protein level (Fig. 6, top panel) and NR5A1 protein level (Fig. 6, middle panel). Another mechanism by which Dex/GR could repress Star transcription is by inhibiting the recruitment of NR4A1 and/or NR5A1 to the proximal Star promoter via protein–protein interactions. In support of this is the fact that activated GR was previously shown to interact with NR4A1 in pituitary corticotrope cells thus preventing its binding to the Pomc promoter (Philips et al. 1997b, Martens et al. 2005) and with NR5A1 in adrenal cells (Ito et al. 1997, Gummow et al. 2006). To begin testing whether Dex/GR could inhibit recruitment of NR4A1 or NR5A1 to the Star promoter, we first performed ChIP followed by quantitative real-time PCR to determine the temporal association of each factor with the proximal Star promoter in MA-10 Leydig cells in response to cAMP stimulation. As shown in Fig. 7A, a significant increase in NR4A1 association with the proximal Star promoter was detected 1 h after cAMP stimulation, peaked at 2 h and decreased at 4 h. A modest but significant increase in NR5A1 recruitment to the proximal Star promoter was observed 2 h post cAMP treatment (Fig. 7A), which is similar to what has been previously reported for NR5A1 (Hiroi et al. 2004). Thus, both NR4A1 and NR5A1 are associated

**Figure 5** Repression of NR4A1/cAMP-dependent activation of the Star promoter by Dex/GR requires an intact −95 bp NbRE/NR5A1 element. Repression of (A) NR4A1- and (B) cAMP-dependent activation of the Star promoter by Dex/GR were studied by co-transfection of MA-10 Leydig cells with a −902 to +17 bp mouse Star reporter construct harbouring a two nucleotide mutation in the −95 bp NbRE/NR5A1 element (Materials and methods) along with either an empty expression vector or expression vectors (250 ng) for NR4A1 and/or GR as indicated. Cells were treated with 100 nM Dex and/or 0.5 mM 8-Br-cAMP for 4 h as indicated before harvesting. The number of experiments, each performed in duplicate, is indicated. Results are shown as fold activation over control (±S.E.M). A different letter indicates a statistically significant difference (P≤0.0022). A schematic of the mutated mouse Star promoter (−902 to +17 bp) with the position of the NR5A1 (grey box), NbRE/NR5A1 (mutation depicted by a large X) and AP-1 (hatched box) elements is shown on top.

**Figure 6** Expression of NR4A1 and NR5A1 is not affected by Dex. MA-10 Leydig cells were treated with vehicle (−), 0.5 mM 8-Br-cAMP and/or 100 nM Dex for 2 and 4 h as indicated. Nuclear extracts were prepared, separated by SDS-PAGE and transferred to PVDF membrane. NR4A1 (top panel) and NR5A1 (middle panel) were immunodetected using antisera specific for NR4A1 and NR5A1. The experiment was repeated thrice and produced identical results. Immunodetection of LMNB1-B (lower panel) was used as a loading control.
with the proximal Star promoter in Leydig cells in a non-exclusive manner. The same methodology was next used to evaluate the impact of Dex/GR on the cAMP-induced recruitment of NR4A1 and NR5A1 to the proximal Star promoter in MA-10 Leydig cells. As shown in Fig. 7B, Dex significantly inhibited by about 55% the cAMP-induced recruitment of NR4A1 to the Star promoter while having no effect on NR5A1 binding to the proximal Star promoter in the absence or presence of cAMP (Fig. 7C). All together, these findings suggest a mechanism where Dex/GR would repress Star transcription, at least in part, by decreasing NR4A1 recruitment to the proximal Star promoter.

### Discussion

Leydig cells normally regulate their intracellular concentration of Gc levels by the action of two Gc metabolizing enzymes, HSD11B1 and -2, both of which are present in these cells (Ge et al. 1997, 2005). Testosterone production can therefore be maintained under normal serum concentrations of Gc that are inactivated by the oxidative activity of HSD11B1 into biologically inert 11-dehydro-Gc (Ge et al. 2005). Under stressful conditions, however, the oxidative capacity of HSD11B1 in Leydig cells is exceeded by high levels of Gc leading to suppression of testosterone biosynthesis (Gao et al. 1996, Ge et al. 1997).

As for many physiological events, several mechanisms, which are not mutually exclusive, have been put forward to explain the effects of GR-dependent stress-mediated decrease in testosterone production by Leydig cells. For instance, Gc were reported to initiate Leydig cell apoptosis (Gao et al. 2002) with the involvement of two transcription factors, NFKB1 and NFATC1 (Wang & Gao 2006, Chai et al. 2007). The nuclear receptor NR4A1 has also been associated with apoptosis in other cell types. Interaction between NR4A1 and the retinoid X receptor (RXR) induces translocation of NR4A1 to the mitochondria where it interacts with BCL2 and converts it from an anti-apoptotic to a pro-apoptotic factor (Katagiri et al. 2000, Cao et al. 2004, Lin et al. 2004). Our current data, however, do not support a role for NR4A1 in the initiation of apoptosis in Leydig cells as a result of Gc exposure since NR4A1 protein levels in the nucleus were normally increased following cAMP stimulation and were not affected by Dex treatment.

Another mechanism by which Gc inhibit steroidogenesis is through repression of genes involved in steroid biosynthesis. Among those is Star that was shown to be repressed by Gc both at the mRNA and protein levels in testicular Leydig cells, ovarian follicles and adrenal cells (Wang et al. 2000, Huang & Shirley 2001, Schwarzenbach et al. 2003, Yu & Li 2006, Fon & Li 2007). We found that Dex, a synthetic Gc, repressed cAMP-induced, but not basal, Star expression in MA-10 Leydig cells, which is in agreement with previous studies reporting that Dex has no effect on basal Star expression (Huang & Shirley 2001, Schwarzenbach et al. 2003). Although the underlying molecular mechanisms remain to be fully elucidated, Bambino & Hsueh (1981) have demonstrated that Dex-mediated repression NR4A1 to the proximal Star promoter.
repression of testosterone production is a GR-dependent event. The importance of GR in the transcriptional repression of Star by Gc was further underscored by the use of RU486, a potent antagonist of Gc action known to bind to GR with high affinity (Beck et al. 1993), which has been shown to stimulate Star expression in testicular Leydig cells (Schwarzenbach et al. 2003) and in the ovary (Huang & Shirley 2001). Activated GR regulates transcription either by binding to GRE in the regulatory region of target genes or through protein–protein interaction with DNA-bound transcription factors (reviewed in Kassel & Herrlich 2007). Consistent with the fact that the Star promoter lacks a consensus GRE (Caron et al. 1997, Wooton-Kee & Clark 2000), we found that part of the Dex-mediated repression of mouse Star promoter activity in Leydig cells involves the nuclear receptor NR4A1, which we have recently identified as a regulator of Star transcription in these cells (Martin et al. 2008).

Our present data suggest that part of the repression is likely mediated through a direct protein–protein interaction between activated GR and NR4A1 into a transcriptionally inactive complex. This is supported by the fact that Dex significantly decreased NR4A1 association with the mouse Star promoter in a native chromatin environment in Leydig cells, while NR5A1 recruitment was unaffected. This was not due to a decrease in transcription factor levels since Dex did not affect NR4A1 and NR5A1 protein levels, which is in agreement with a previous report for NR5A1 (Yu & Li 2006). A similar transcriptionally inactive GR/NR4A1 interaction has been previously reported in pituitary corticotrope cells, where Gc repress expression of the Pome gene (Philips et al. 1997b, Martens et al. 2005). Another mechanism by which Dex could repress cAMP-induced Star transcription is through the modulation of coactivator/corepressor recruitment. Dex was recently reported to inhibit the cAMP-mediated increase in the association of acetylated histone H3, a marker of active gene transcription, with the proximal Star promoter (Christenson et al. 2001, Hiroi et al. 2004, Fon & Li 2007). Furthermore, inhibition of Star mRNA and protein levels in the testis of Dex-treated rats was found to be correlated with increased expression of the atypical nuclear receptor NR0B1 (Yu & Li 2006), which is known to be associated with corepressors and to repress gene expression (Crawford et al. 1999, Altincicek et al. 2000). NR0B1 can also interact with NR4A1 and NR5A1, both activators of Star expression, leading to the repression of their transactivation properties (Ito et al. 1997, Song et al. 2004, Yu & Li 2006). Expression of GIOT-1, another repressor found in Leydig cells, is upregulated by NR4A1 in response to LH/cAMP (Song et al. 2006). GIOT-1 was shown to act as a repressor of NR5A1-mediated activation of the Cyp17a1 promoter (Song et al. 2006). It is therefore possible that the NR0B1 and GIOT-1 repressor, through their interaction with NR4A1 and NR5A1, might also contribute to the Dex-mediated inhibition of cAMP-induced Star expression in Leydig cells.

Although we found a role for NR4A1 in Dex-mediated repression of cAMP-induced Star transcription, other transcription factors are also involved in this process. This is supported by the fact that the Dex-mediated repression was more important when 8-Br-cAMP was used compared with NR4A1 that is consistent with the fact that cAMP activates several transcription factors in addition to NR4A1 in Leydig cells. Additional studies are required to identify these factors to ultimately better understand how stress inhibits Star gene expression and testosterone production in Leydig cells.

Declaration of interest

The authors declare that there is no conflict of interest that would prejudice their impartiality.

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