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.

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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 (Charpenet *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 pseudohermaphroditism and lipoid 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).

NR4A1 (NUR77, NGFI-B), along with NR4A2 (NURR1) and NR4A3 (NOR1), form a family of transcription factors that are characterized as immediate early response genes (Giguere 1999, Eells *et al.* 2000, Maxwell & Muscat 2005). Their expression is rapidly and strongly induced by various stimuli in numerous tissues, including hormonally stimulated steroidogenic cells (Davis & Lau 1994, Park *et al.* 2001, 2003, Song *et al.* 2001, Li *et al.* 2004, Martin & Tremblay 2005). NR4A1 transactivation potential is also modulated by post-translational modifications (Davis *et al.* 1993, Hirata *et al.* 1993, Li & Lau 1997, Wingate & Arthur 2006) and/or modulation of its intracellular localization in response to certain stimuli (Klopotowska *et al.* 2005). NR4A1 binds DNA as a monomer to the sequence AAAAGGTCA, called NR4A1 response element (NBRE), that is very similar to that recognized by the nuclear receptor steroidogenic factor 1 (NR5A1, SF1; Wilson *et al.* 1993), an essential regulator of expression of many steroidogenic genes (reviewed in Parker *et al.* 2002, Val *et al.* 2003). In Leydig cells, NR4A1 has been found to regulate genes involved in steroidogenesis (Zhang & Mellon 1997, Bassett *et al.* 2004, Hong *et al.* 2004, Havelock *et al.* 2005, Martin & Tremblay 2005), including *Star* (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 GGTCAnnnTGTTCT 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 (CATCCTTGA 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). Immuno-detection 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 Transcriptor 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. PCRs were performed using the following *Star*-specific primers: forward, 5'-TTG GGC ATA CTC AAC AAC CA-3' and reverse, 5'-CCT TGA CAT TTG GGT TCC AC-3'. As an internal control, PCRs were performed using previously described *Rpl19*-specific primers (Guigon *et al.* 2005). The PCRs were done using the following conditions: 10 min at 95 °C followed by 35 cycles of denaturation (5 s at 95 °C), annealing (5 s at 62 °C for both *Rpl19* and *Star* cDNAs) and extension (20 s at 72 °C) with single acquisition of fluorescence at the end of each extension steps. The specificity of PCR products was confirmed by analysis of the melting curve and agarose gel electrophoresis. Quantification of gene expression was performed using the Relative Quantification Software (Roche Diagnostics) and is expressed as a ratio of *Star* to *Rpl19* mRNA levels.

Chromatin immunoprecipitation (ChIP) assay

ChIP assays were performed as described previously (Robert *et al.* 2006). The genomic DNA immuno-precipitated 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 ACT 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.

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

Figure 1 Dex inhibits cAMP-induced *Star* expression in Leydig cells. MA-10 Leydig cells were treated with 8-Br-cAMP (0.5 mM) in the presence or absence of Dex (100 nM) for the indicated times and total RNA was isolated and used in quantitative real-time PCR using primers specific for *Star* cDNA as described in Materials and methods. Results were corrected with the *Rpl19* cDNA. Results are the mean of three individual experiments performed in duplicate ($\pm$ S.E.M.). * $P < 0.03$.

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 GR 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

Figure 2 Dex represses NR4A1 and cAMP-induced *Star* promoter activity. Repression by Dex of the (A) NR4A1- and (B) cAMP-dependent activation of the *Star* promoter. MA-10 Leydig cells were co-transfected with a -902 to +17 bp mouse *Star* reporter construct along with either an empty expression vector or an expression vector (250 ng) for NR4A1 as indicated. Cells were treated with 100 nM Dex and/or 0.5 mM 8-Br-cAMP 4 h before harvesting. The number of experiments, each performed in duplicate, is indicated. Results are shown as fold activation over control ($\pm$ S.E.M.). A different letter indicates a statistically significant difference. In (A), $P < 0.0001$ except between groups b and c where $P = 0.0035$. In (B), $P \leq 0.0002$ except between groups b and c, where $P = 0.0481$ and groups d and b where $P = 0.0318$. A schematic of the mouse *Star* promoter (-902 to +17 bp) with the position of the NR5A1 (grey box), NBRE/NR5A1 (black box) and AP-1 (hatched box) elements is shown on top.

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 NBRE/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 NBRE/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

Figure 3 Effect of GR on the Dex-mediated repression of *Star* promoter activity. Repression by Dex of the (A) NR4A1- and (B) cAMP-dependent activation of the *Star* promoter is studied in the presence of GR. MA-10 Leydig cells were co-transfected with a -902 to +17 bp mouse *Star* reporter construct 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 as indicated for 4 h before harvesting. The number of experiments, each performed in duplicate, is indicated. Results are shown as fold activation over control ($\pm$ s.e.m.). A different letter indicates a statistically significant difference. In (A), $P < 0.0001$ except between groups b and c where $P = 0.0291$. In (B), $P < 0.0001$ except between groups b and c where $P = 0.0108$. A schematic of the mouse *Star* promoter (-902 to +17 bp) with the position of the NR5A1 (grey box), NBRE/NR5A1 (black box) and AP-1 (hatched box) elements is shown on top.

Figure 4 The minimal *Star* promoter is unresponsive to Dex/GR. MA-10 Leydig cells were co-transfected and treated as described in Fig. 2 (for A and B) and in Fig. 3 (for C and D) except that a -71 to +17 bp mouse *Star* reporter construct was used. The number of experiments, each performed in duplicate, is indicated. Results are shown as fold activation over control ($\pm$ s.e.m.). A different letter indicates a statistically significant difference. In (A) $P \leq 0.0499$ and in (B) $P \leq 0.0044$. A schematic of the mouse *Star* promoter (-71 to +17 bp) with the position of the remaining NR5A1 (grey box) element is shown on top.

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 -95 bp behaved exactly as the minimal -71 bp *Star* reporter (Fig. 4). Altogether, these results indicate that an intact NBRE/NR5A1 element at -95 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*

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 ($\pm$ S.E.M.). A different letter indicates a statistically significant difference ($P \leq 0.0022$). 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.

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 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 (lower panel) was used as a loading control.

Figure 7 Recruitment of NR4A1 to the proximal *Star* promoter is inhibited by Dex. (A) Time-dependent recruitment of NR4A1 and NR5A1 to the proximal *Star* promoter in response to cAMP. MA-10 Leydig cells were treated with 0.5 mM 8-Br-cAMP for the indicated time and ChIP assays were performed. Chromatin was immunoprecipitated with either a NR4A1 (square) or NR5A1 (triangle) antiserum. An IgG was used as control (diamond). For a given time point, asterisks indicate a statistically significant difference in cAMP between treated and untreated. * $P < 0.0001$, ** $P = 0.0023$, *** $P = 0.0275$. (B and C) Dex inhibits NR4A1 recruitment to the *Star* promoter. Association of (B) NR4A1 and (C) NR5A1 to the proximal mouse *Star* promoter was determined by ChIP assay using MA-10 Leydig cells treated with vehicle (–), 0.5 mM 8-Br-cAMP and/or 100 nM Dex as indicated. A different letter indicates a statistically significant difference. In (B), $P \leq 0.0003$ except between groups a and c where $P = 0.0022$. In (C), $P = 0.0024$. In (A), (B) and (C), a 258 bp fragment of the proximal *Star* promoter encompassing the –95 bp NBRE/NR5A1 element was amplified by real-time PCR immediately following the ChIP assay. An aliquot (5%) of chromatin preparation before immunoprecipitation (input) was used as positive control. Results are presented as a ratio of *Star* promoter immunoprecipitated DNA to input DNA levels from three independent experiments ($\pm$ S.E.M.).

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, NF κ B1 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 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, Wootton-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 *Pomc* 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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