Effects of ACTH and expression of the melanocortin-2 receptor in the neonatal mouse testis

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Abstract

ACTH has been shown to stimulate androgen production by the fetal/neonatal mouse testis through the melanocortin type 2 receptor (MC2R). This study was designed to localize the expression of MC2R in the neonatal mouse testis and characterize the effects of ACTH on testicular androgen production. Using immunohistochemistry, MC2R was localized to the fetal-type Leydig cell population of the neonatal testis. ACTH caused a time-dependent increase in cyclic AMP (cAMP) and testosterone production by isolated cells with an increase in cAMP apparent in < 3 min. There was no additive effect of maximally stimulating doses of ACTH and human chorionic gonadotropin (hCG). Androgen production in response to ACTH and hCG was reduced by UO126 and dexamethasone, which are the inhibitors of ERK1/2 and phospholipase A2 respectively. Expression of mRNA encoding STAR was increased fourfold by both ACTH and hCG, although expression of mRNA encoding for steroidogenic enzymes was not markedly affected. The potency of N-terminal fragments of ACTH to stimulate androgen production was similar to that seen previously in the adrenal. Data indicate that both LH and ACTH, acting through their respective receptors, stimulate steroidogenesis by fetal-type Leydig cells via arachidonic acid, protein kinase A, and ERK1/2 activation of STAR.

Introduction

Adrenocorticotropic hormone (ACTH) regulates corticosteroid output from the adrenal cortex through interaction with the melanocortin type 2 receptor (MC2R). Outside of the adrenal cortex, MC2R expression has been described previously in murine adipocytes (Cammas et al. 1997) and we have shown recently that the receptor is also expressed in the mouse fetal testis (O’Shaughnessy et al. 2003). The testicular receptors appear to be functionally active since ACTH will stimulate testosterone production by the fetal and neonatal mouse testis which both contain fetal-type Leydig cells although the effects are lost in the postpubertal animal (O’Shaughnessy et al. 2003). Androgen production by the fetal and neonatal mouse testis is dependent upon the fetal population of Leydig cells, which persists into early neonatal life and is responsive to luteinizing hormone (LH; O’Shaughnessy et al. 2005). The effects of ACTH on fetal/neonatal androgen production could be directly mediated through melanocortin receptors on the fetal-type Leydig cells or indirectly mediated through another cell type. This second possibility is supported by the presence of paracrine factors in the fetal testis, which stimulate Leydig cell function (El-Gehani et al. 1998, 2001). This study was designed, therefore, to localize expression of MC2R in the neonatal testis (which contains fetal-type Leydig cells) and, thereafter, to characterize the direct effects of ACTH stimulation of the testis.

Materials and Methods

Animals

Normal mice were bred at the University of Glasgow Veterinary School and maintained as required under United Kingdom Home Office regulations. The mice used were derived from F1 hybrids of C3H/HeH and 101/H strains. For all studies, the animals were aged 2 or 3 days after birth with the day of birth designated as day 1.

Cell isolation and incubation

Dispersed testicular cells were prepared by collagenase treatment of whole testes as previously described (Stalvey & Payne 1983). The testes from four to eight animals were dispersed at 37 °C in DMEM/F12
containing 1 mg/ml collagenase (Worthington CLS type 4, purchased from Lorne Laboratories Ltd, Twyford, UK; Stalvey & Payne 1983) and isolated cells were filtered through a nylon sieve with a pore size of 50 μm. The proportion of Leydig cells in the cell preparations was 3–5% when tested by staining for 3β-hydroxysteroid dehydrogenase activity (Payne et al. 1980) although this parameter was not routinely measured in all samples. Aliquots of isolated cells (1 ml total) were incubated for up to 18 h at 37 °C in DMEM/F12 in an atmosphere of 5% CO₂ and in the presence of varying concentrations of human chorionic gonadotropin (hCG) or ACTH peptide fragments (Sigma–Aldrich Co Ltd). In experiments designed to measure cAMP production, isobutyl methyloxanthine (Sigma–Aldrich) was included in the incubation medium at a concentration of 0.1 mM. In experiments to study the role of extracellular signal-regulated kinase ERK or arachidonic acid (AA) in the steroidogenic response to tropic hormone stimulation, the cells were preincubated for 30 min with UO126 (Calbiochem, Merck Biosciences Ltd, Nottingham, UK), which is a specific inhibitor of ERK (Davies et al. 2000) or dexamethasone, which is a phospholipase A2 (PLA₂) inhibitor (Blackwell et al. 1978). Tropic hormone was added after the preincubation period and the cells were incubated with inhibitor and hormone together for 3 h.

At the end of the incubation period, cells and medium were placed in a heating block at 100 °C for 5 min and the medium collected after centrifugation at 4000 g for 10 min. To measure the changes in gene expression, cells and medium were separated at the end of the incubation by centrifugation at 15000 g and the cell pellet stored in liquid N₂.

**RT and real-time PCR**

Total RNA was extracted using Trizol (Life Technologies). Isolated RNA was reverse transcribed using random hexamers and Moloney murine leukemia virus reverse transcriptase (Superscript II, Invitrogen) as described previously (O'Shaughnessy & Murphy 1993, O'Shaughnessy et al. 1994).

To quantify the content of specific mRNA species in testicular cells following incubation in vitro with hCG or ACTH, a real-time PCR approach was used, which utilized the SYBR green method following RT of the isolated RNA. Real-time PCRs were performed in a 96-well plate format using a Stratagene MX3000 cycler. Reactions contained 5 μl of 2×SYBR mastermix (Stratagene, Amsterdam, The Netherlands), primer (100 nM), and template in a total volume of 10 μl. The thermal profile used for amplification was 95 °C for 8 min followed by 40 cycles of 95 °C for 20 s, 63 °C for 20 s, and 72 °C for 30 s. At the end of the amplification phase, a melting curve analysis was carried out on the products formed. Negative controls without RNA were included in the RT and subsequent real-time PCR studies.

Primers for real-time PCR were designed using parameters previously described (Czechowski et al. 2004). The genes studied and primers used were:

**Williams-Beuren syndrome chromosome region1 (WBscr1)**

- **Forward** agcatacggagctctgcttggttc
- **Reverse** tcacccaaggtgcaccgtcataa

**Cytochrome P450 side chain cleavage (Cyp11a1)**

- **Forward** cacagacgcatcaagcagcaaaa
- **Reverse** gcattgataacgccgtgccc

**Cytochrome P450 17α-hydroxylase (Cyp17)**

- **Forward** ttgctcccatcttcctcgcctg
- **Reverse** aggccagcgcgtttcttg

**Star**

- **Forward** cgtcggagctcttgcttggttc
- **Reverse** tcgtccctctcctgcctgc

Expression of each mRNA species was determined relative to the house-keeping gene wbscr1 as previously described (O’Shaughnessy et al. 2002).

**Immunohistochemistry**

Neonatal testes were fixed in 4% paraformaldehyde for 1 h, then washed in 70% ethanol, dehydrated, and embedded in paraffin. Sections (5 μm) were mounted on glass slides, dewaxed, and rehydrated. Endogenous biotin was blocked using an avidin/biotin blocking kit (R&D systems Europe Ltd, Abingden, UK) and sections were incubated with primary antibody overnight at 4 °C. The antibodies used were rabbit anti-mouse MC2R (Alpha Diagnostic, supplied by Autogen Bioclear, Calne, Wiltshire, UK) and rabbit anti-bovine CYP11A1 (gift from AH Payne). Sections were washed and incubated for 30 min with biotinylated secondary antibody (R&D systems Europe Ltd). Bound antibody was visualized using 3,3-diaminobenzidine tetrahydrochloride (R&D systems Europe Ltd). Negative controls without the primary antibody were included in each experiment.

**RIA**

Levels of testosterone in incubation medium were measured by RIA as previously described (O’Shaughnessy & Sheffield 1990). Levels of cAMP were measured using a commercial assay system (Amersham Biosciences).
Statistical analysis

Changes in cAMP levels over time were analyzed by one-tailed t-tests as basal levels were undetectable. Other statistical analysis was by two-factor ANOVA using log-transformed data followed by individual t-tests using the pooled error from the initial analysis.

Results

Localization of MC2R expression in neonatal testis

Using immunohistochemistry, expression of MC2R in the neonatal testis was limited to the interstitial tissue (Fig. 1A). Labeling of an adjacent section with the Leydig cell marker CYP11A1 indicated that the same cells were expressing both MC2R and CYP11A1 (Fig. 1B). A small number of cells appeared to express CYP11A1 but not MC2R and this may be an indication that not all fetal-type Leydig cells express MC2R. All the cells expressing MC2R appeared to express CYP11A1. No immunohistochemical staining for MC2R was seen in the adult testis (not shown).

Testicular response to ACTH

Incubation of isolated neonatal testicular cells with ACTH or hCG led to a time-dependent increase in testosterone production in response to both hormones (Fig. 2A). Responses to ACTH and hCG were identical up to 7 h and varied little up to 18 h (Fig. 2A). The response to ACTH was rapid with an increase in testosterone production seen within 5 min (Fig. 2B). Changes in cAMP levels in response to ACTH were also very rapid with an increase in cyclic nucleotide apparent within 3 min (Fig. 2C). To determine whether ERKs1/2 are involved in the steroidogenic response of fetal-type Leydig cells to ACTH and hCG, cells were incubated with the specific inhibitor UO126. In the presence of UO126, there was a reduction in basal androgen production and both ACTH- and hCG-stimulated production were also significantly reduced (Fig. 3). The role of PLA2 in the steroidogenic response of cells to ACTH and hCG was measured using the PLA2 inhibitor dexamethasone.

Figure 1 Immunohistochemical localization of (A) MC2R and (B) CYP11A1 in the neonatal testis. Adjacent sections of testes (5 μm) from a neonatal mouse (day 2) were incubated with antibody to MC2R or CYP11A1 and visualized following binding to a biotinylated secondary antibody. Results show that the same cells in adjacent sections are stained with both antibodies (arrows). Control sections with no primary antibody did not show staining (inset to A).

Figure 2 Time-dependent changes in testosterone and cAMP production by isolated cells from the neonatal testis in response to ACTH or hCG. Cells were isolated from neonatal testes and incubated with ACTH (10⁻⁷ M) or hCG (10⁻⁹ M). (A) Testosterone production during long-term incubation with ACTH or hCG. (B and C) Testosterone and cAMP production during short-term incubation in the presence of ACTH (10⁻⁷ M). *P<0.05 compared with basal level at same time. These studies have been repeated once.

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Basal steroidogenesis was not affected by dexamethasone although both ACTH- and hCG-stimulated production was inhibited at the highest dose of dexamethasone (Fig. 3). Steroidogenesis in the presence of 22R-hydroxycholesterol (22ROHC), which bypasses the second messenger systems to deliver cholesterol directly to the mitochondrion, was unaffected by the presence of UO126 or dexamethasone (Fig. 3).

**Effect of ACTH peptide fragments on Leydig cell steroidogenesis**

To determine whether the Leydig cell MC2R shows the same relative sensitivity to ACTH peptide fragments as the adrenal MC2R (Vinson et al. 1986, Hinson & Birmingham 1987) neonatal testicular cells were incubated with ACTH (1–24), (1–17) and (1–16; Fig. 4A). The cells were most responsive to ACTH (1–24) with responsiveness to ACTH (1–17) and (1–16) about 100- and 1000-fold less respectively. Addition of maximally stimulating doses of both ACTH and hCG caused no further increase in testosterone production when compared with each hormone alone indicating that there was no apparent additive effect of the hormones once the cells were maximally stimulated (Fig. 4B).

**Acute effects of ACTH and hCG on fetal Leydig cell gene expression**

To determine whether hCG and ACTH stimulate similar changes in short-term gene expression in the neonatal testis, isolated cells were incubated with hCG or ACTH for 5 h and expression of three key Leydig cell genes measured. In cells incubated with ACTH or hCG, there was a small but significant increase in *Cyp11a1* mRNA levels, no significant change in *Cyp17* and a fourfold increase in *Star* mRNA levels (Fig. 5). There was no difference in the response of the cells to hCG or ACTH.
During development, two populations of Leydig cells arise sequentially. The fetal-type Leydig cells arise soon after testis differentiation in the mouse and are subsequently replaced by the adult population, which starts to develop in the postnatal, prepubertal period (Baker et al. 1999, Nef et al. 2000). The adult Leydig cell population is primarily responsive to LH and, in the absence of LH, cell numbers fail to develop and androgen production is minimal (Cattanach et al. 1977, Baker & O’Shaughnessy 2001, Ma et al. 2004). The fetal Leydig cells are also responsive to LH but, unlike the adult population, LH is not essential for fetal Leydig cell development and function (O’Shaughnessy et al. 1998, Zhang et al. 2004). Results from this study now show that the fetal-type Leydig cells are also directly responsive to ACTH. In contrast, the postpubertal testis is not responsive to ACTH and MC2R expression is minimal indicating that ACTH has no effect on the adult Leydig cell (O’Shaughnessy et al. 2003). In contrast to the mouse, earlier studies have reported that adult rabbit and guinea pig testes (but not rat, dog or hamster) will increase testosterone in response to ACTH (Juniewicz et al. 1988). This suggests that the adult Leydig cells may also express MC2R in these species or there is persistence of significant numbers of fetal Leydig cells into the adult testes.

It is well established that cAMP is a critical second messenger in the LH-dependent stimulation of Leydig cell steroidogenesis. It has, for example, been shown that LH/hCG causes rapid stimulation of cAMP production and that dibutylryl cAMP stimulates testosterone production through activation of StAR protein (Saenz 1994, Stocco 2001). Results from this study show that ACTH causes a rapid stimulation of cAMP production within 3 min and before testosterone production is apparent. It is likely, therefore, that both LH/hCG and ACTH stimulate steroidogenesis through cAMP-dependent pathways. It is known that cAMP activates protein kinase A (PKA), which in turn, activates StAR protein (Stocco 2001). Recent studies suggest, however, that cAMP activation of ERK1/2 might also play a role in stimulation of adult Leydig cell steroidogenesis (Martinelle et al. 2004). Using the specific ERK inhibitor UO126, our data show that the transduction of signal from the LH-receptor or MC2R through the ERK cascade contributes to steroidogenic activity in the fetal mouse Leydig cell. Effects of UO126 were only seen at the higher concentrations of inhibitor, but this is similar to the effects of UO126 on rat Leydig cells (Martinelle et al. 2004) and reflects sensitivity of ERK1/2 to UO126 inhibition.

In addition to the pathways described above, there is also abundant evidence that AA can mediate tropic hormone signal transduction in steroidogenic cells (Stocco et al. 2005). Thus, G protein or cAMP can activate PLA2, which in turn, catalyzes AA release from phospholipids and leads to the activation of StAR (Wang et al. 2000). It has been shown that LH can induce AA release in rat Leydig cells and inhibition of AA release from phospholipids causes a marked inhibition of steroidogenesis (Abayasekara et al. 1990, Cooke et al. 1991). Our data

Discussion

Five types of melanocortin receptor have been identified to date, each with characteristic affinity for the different melanocortins derived from proopiomelanocortin (Abdel-Malek 2001). In previous studies, we have shown that ACTH acts on the testis through MC2R (O’Shaughnessy et al. 2003) and the immunohistochemical data presented here show that MC2R expression is restricted to the interstitial tissue and to cells expressing CYP11A1. Since CYP11A1 expression in the testis is restricted to the Leydig cells (Wang et al. 2003), it is clear that MC2R is a Leydig cell product. It has been reported recently that MC2R is expressed in both the gonocytes and the interstitial tissue of the fetal mouse testis (Nimura et al. 2006). Our immunohistochemical and functional data agree with the interstitial localization of MC2R and suggest strongly that the Leydig cells are the site of interstitial expression. We did not, however, observe staining of MC2R in the germ cell component. There is no clear explanation for this discrepancy although the animals used in this study were about 3 days older than those used previously (Nimura et al. 2006) and it is possible that gonocyte expression is lost as the cells move into the neonatal phase of proliferation (Baker & O’Shaughnessy 2001). Other studies have shown that ACTH can stimulate aromatase activity in neonatal Sertoli cells suggesting MC2R could be expressed in these cells (Boitani et al. 1989). Sertoli cells were, however, equally responsive to other melanocortins (α-melanocyte-stimulating hormone (MSH) and βMSH) indicating that this effect is not mediated through MC2R but another melanocortin receptor (Abdel-Malek 2001).
using the PLA₂ inhibitor dexamethasone show that a component of the stimulatory effect of LH and ACTH on androgen production by the fetal Leydig cells may be mediated through AA release from phospholipid. The effects on hCG- and ACTH-stimulation were, however, only seen at relatively high concentrations of inhibitor, higher than those required to show inhibition of steroidogenesis in MA-10 mouse Leydig tumor cells (Wang et al. 2000). Glucocorticoids can have direct inhibitory effects on Leydig cell steroidogenesis through the activation of the glucocorticoid receptor (Bambino & Hsueh 1981, Hales & Payne 1989), although this is unlikely in our studies because of the timescale of the effects and because steroidogenesis was normal in the presence of 22ROHC. Nevertheless, the relative insensitivity of fetal Leydig cells to dexamethasone may be an indication that the pathway through PLA₂ is relatively minor in these cells.

In the adrenal, ACTH acts primarily through the stimulation of cAMP, PKA, and activation of STAR although there is good evidence that ACTH can act directly or downstream through the release of AA (Cooke 1999). There is also evidence of activation through ERK1/2 (Ferreira et al. 2004) although this pathway is less well documented in the adrenal. Activation of these pathways by ACTH in the fetal-type Leydig cells is, therefore, consistent with the effects of ACTH mediated through MC2R in the adrenal.

The signal pathways outlined above act to increase the activity of preformed STAR and also act to increase transcription of the Star gene (Stocco et al. 2005). Thus, increased expression of Star mRNA in neonatal mouse testis by hCG and ACTH is consistent with both hormones acting through similar signaling pathways in mouse fetal Leydig cells. It is known that LH acts to regulate and maintain expression of Cyp11a1 in the testis (Malaska & Payne 1984, Mason et al. 1984, Scott et al. 1990) and studies using MA-10 cells have shown that the effects of LH can be rapid (Mellon & Vaisse 1989). The effects of LH and ACTH on Cyp11a1 in the isolated testicular cells reported here are consistent, therefore, with these earlier studies. Expression of Cyp17 in the Leydig cell has also been shown to be regulated by LH (Anakwe & Payne 1987, Baker et al. 2003) and the effects of cAMP on expression have been shown to be rapid in MA-10 cells (Laurich et al. 2002). It is not clear, therefore, why no increase in Cyp17 was seen in the present study although it is possible that increasing androgen levels in the medium may have inhibited Cyp17 expression (Hales et al. 1987).

Results from this study show that the fetal Leydig cells are responsive to both LH and ACTH and both hormones appear to act on the cell through similar mechanisms. In animals lacking either LH (or LH-receptor) or ACTH, fetal testosterone production is normal (O'Shaughnessy et al. 1998, 2003, Ma et al. 2004, Zhang et al. 2004) although studies using the T/erb-b null mouse indicate that the fetal pituitary is required for normal fetal Leydig cell function (Pakarinen et al. 2002). It is possible, therefore, that LH and ACTH may act to regulate Leydig cell function in a redundant fashion.

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