Oxysterols regulate expression of the steroidogenic acute regulatory protein

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Abstract

The steroidogenic acute regulatory (StAR) protein promotes intramitochondrial delivery of cholesterol to the cholesterol side-chain cleavage system, which catalyzes the first enzymatic step in all steroid synthesis. Intriguingly, substrate cholesterol derived from lipoprotein can upregulate *StAR* gene expression. Moreover, substrate oxysterols have been suggested to also play a role. To investigate whether oxysterols can regulate StAR expression, two steroidogenic cell lines, mouse Y1 adrenocortical and MA-10 Leydig tumor cells, were treated with various oxysterols and steroids, including 25-hydroxycholesterol (25 OHC), 22(R)OHC and 20 α OHC. The majority of these compounds rapidly increased StAR protein levels within as little as 1 h. The most potent oxysterols were 20 α OHC for Y1 and 25 OHC for MA-10 cells. After 8 h, StAR mRNA abundance also increased whereas there were no detected changes in promoter activity. Thus, in contrast to lipoprotein, oxysterols acutely increase StAR protein levels independently of mRNA abundance, and later increase mRNA levels independently of new gene transcription. Therefore, we propose that oxysterols modulate steroidogenesis at two levels. First, oxysterols may be important in post-transcriptional regulation of StAR activity and production of steroids for paracrine action. Secondly, through direct conversion to steroid, oxysterols may account in part for StAR-independent steroid production in the body.

Journal of Molecular Endocrinology (2004) **32**, 507–517

Introduction

The steroidogenic acute regulatory (StAR) protein is a critical mitochondrial protein, which mediates the rate-limiting step in steroidogenesis (Stocco 2001). Upon trophic hormone stimulation, StAR is rapidly synthesized as a larger precursor in steroidogenic cells and, prior to or during mitochondrial import and processing, promotes the transfer of cholesterol from the outer to the inner mitochondrial membrane and the resident steroidogenic machinery (Krueger & Orme-Johnson 1983, Clark *et al.* 1994, Bose *et al.* 1999, 2002). There, cholesterol is oxidized and converted to steroid by cytochrome P450 scc, which is part of the cholesterol side-chain cleavage system. Thus,

continual *de novo* synthesis of StAR ensures uninterrupted availability of cholesterol substrate to P450 scc for maximal steroid production. Mutations in StAR that compromise its activity give rise to congenital lipid adrenal hyperplasia, with affected individuals showing a block in steroid synthesis and a pathological accumulation of cholesterol in their adrenal glands (Lin & Sugwara 1995, Bose *et al.* 1996, Miller 1997). A similar phenotype is observed in mice in which the *StAR* gene is disrupted (Caron *et al.* 1997b).

The control of StAR expression is complex, involving both transcriptional and post-transcriptional mechanisms. Interestingly, elevations in cholesterol supplies derived from lipoprotein increase StAR mRNA and protein levels (Reyland

et al. 2000), suggesting that substrate availability and steroid synthesis are linked in part through regulation of StAR activity. Other evidence indicates that StAR protein levels are also positively regulated by oxysterols. These compounds are generated from oxidation of cholesterol by specific hydroxylases that are present in many cell types in the body, including steroidogenic cells (Rennert *et al.* 1990, Russell 2000). Oxysterols regulate cholesterol and lipid homeostasis through, for instance, the transcription factors liver X receptor α and sterol regulatory element binding protein (SREBP) cleavage-activating protein (Edwards *et al.* 2002, Horton *et al.* 2002, Repa *et al.* 2002).

More hydrophilic than cholesterol, side-chain oxygenated sterols readily pass across lipophilic membranes and can translocate by diffusion into the mitochondria to P450 scc for direct conversion to steroid in the absence of StAR activity (Jefcoate *et al.* 1974, Meaney *et al.* 2002). However, oxysterols may also influence the synthesis of steroid from cholesterol, potentially through steroidogenic factor-1 (SF-1), an important transcription factor for *StAR* and steroidogenic enzyme gene expression (Lala *et al.* 1997, Mellon & Bair 1998). Recently, it was shown that chronic treatment with oxysterols may also influence StAR protein levels in human luteinized granulosa cells in the absence of changes in mRNA (Christenson *et al.* 1998). The present studies were undertaken to investigate how oxysterols regulate the expression of StAR in steroidogenic cells. We show here that treatment of Y1 adrenocortical or MA-10 Leydig tumor cells with oxysterols results in a biphasic increase in StAR protein expression. Oxysterols cause an acute increase in StAR protein abundance that is independent of changes in StAR mRNA, but requires new protein synthesis. This acute increase is followed by a chronic increase in StAR protein that correlates with an elevation of StAR mRNA levels, but is independent of new *StAR* gene transcription.

Materials and methods

Cell culture

The stable Y1 subclone, BS1, was a generous gift of Dr Bernard P Schimmer (University of Toronto, ON, Canada) (Schimmer 1979). Cells were maintained in Ham's F10 medium supplemented

with 100 U/ml penicillin, 100 mg/ml streptomycin, 2 mM L-glutamine, 12.5% heat-inactivated horse serum, and 2.5% heat-inactivated fetal calf serum. Subconfluent cells were serum-starved by pre-incubation for 20–24 h in minimal media (Ham's F10 media supplemented with 0.5% BSA and 1% Nutridoma SP from Roche, Indianapolis, IN, USA) prior to the addition of sterol. All cell culture reagents were purchased from Invitrogen (Grand Island, NY, USA). The mouse MA-10 Leydig tumor cell line was a gift of Dr Mario Ascoli (University of Iowa, Iowa City, IA, USA). Cells were maintained in Waymouth's MB 752/1 medium with 15% horse serum at 37 °C, in 5% CO₂ as previously described (Ascoli *et al.* 1987). Cells were treated with dibutyryl cAMP (dbcAMP) (Sigma-Aldrich, St Louis, MO, USA), low-density lipoprotein (LDL) (Biomedical Technologies Inc., Stoughton, MA, USA) or sterols or steroids (Steraloids Inc., Newport, RI, USA) dissolved in ethanol (EtOH). The final concentration of EtOH in media never exceeded 0.1%. Cycloheximide was purchased from Sigma-Aldrich.

Northern blot analysis

RNA was purified using RNA Stat-60 (Tel-Test Inc., Friendswood, TX, USA) for Y1 cells or TRIzol reagent (Invitrogen) for MA-10 cells according to the manufacturers' directions. Ten micrograms of total RNA were separated on a 1.2% agarose gel containing 2.2 M formaldehyde, transferred to Nytran (Scheicher and Schuell, Keene, NH, USA), crosslinked with ultraviolet light using a Stratalinker (Stratagene, La Jolla, CA, USA), and hybridized to the indicated cDNA probe. cDNA probes were prepared by random priming in the presence of [α -³²P]dCTP (Amersham Biosciences, Piscataway, NJ, USA) using mouse *StAR* cDNA and rat glyceraldehyde 3-phosphate dehydrogenase (GAPDH) cDNA (gift of Dr Kenneth B Marcu, State University of New York, Stony Brook, NY, USA). 18S rRNA probes were also labeled by random priming (Prime-It II; Stratagene) using [α -³²P]dCTP (EasyTides; Perkin Elmer, Boston, MA, USA). Hybridization was performed essentially as previously described (Walsh & Stocco 2000). Levels of 18S rRNA, GAPDH and *StAR* mRNA were quantified using a Molecular Dynamics phosphorimager system (Amersham) for Y1 cells or by densitometry of

autoradiograms using the Visage 2000 computer-assisted image analysis system (BioImage, Ann Arbor, MI, USA) for MA-10 cells.

Immunoblot analysis

StAR protein expression was detected by immunoblot analysis as previously described using 50 µg whole Y1 cell protein and 25 or 50 µg mitochondrial protein from MA-10 cells (Wang *et al.* 1998, Reyland *et al.* 2000). Two different antisera generated against recombinant N62-truncated human StAR were generously provided by Drs Jerome F Strauss III (University of Pennsylvania Medical Center, Philadelphia, PA, USA) and Walter L Miller (University of California at San Francisco, CA, USA) and used to detect StAR protein in Y1 and MA-10 protein blots respectively (Pollack *et al.* 1997, Bose *et al.* 1999). Immunoreactive bands were visualized by chemiluminescence using ECL (Amersham) or Renaissance (Perkin Elmer) kits and were quantified by phosphorimaging or densitometry.

Transient expression from the StAR promoter

To investigate effects of oxysterols on StAR promoter activity, Y1 cells were co-transfected using the calcium phosphate method (Wigler *et al.* 1977) with a 966 bp fragment of the mouse StAR promoter (p-966 StAR/luc) (gift of Dr Keith L Parker, University of Texas Southwestern Medical Center, Dallas, TX, USA) (Caron *et al.* 1997a) or a 1.3 kb fragment of the human StAR promoter (pGL1.3 kbStAR) (kindly supplied by Dr Strauss) (Sugawara *et al.* 1996) cloned into luciferase gene reporter constructs and pCMV(β-galactosidase) as a control for transfection efficiency. The cells were incubated with the DNA/calcium phosphate mixture for 18 h, washed with PBS, and further incubated in minimal media for 24 h. Cells were then treated with sterols or LDL and harvested. Luciferase and β-galactosidase activities were assayed as previously described (Reyland *et al.* 1998).

Results

Oxysterols increase StAR protein levels in steroidogenic cells

To establish whether oxysterols affect the expression of StAR, we first investigated the effects

of three different hydroxycholesterols (OHCs), 20αOHC, 22(R)OHC and 25 OHC, on two different steroidogenic cell lines: Y1 adrenocortical cells and MA-10 Leydig tumor cells. All three oxysterols increased StAR protein levels in a concentration-dependent manner in Y1 cells (Fig. 1). The minimum effective concentration was 1 µg/ml. In cells treated with 20αOHC or 22(R)OHC, the maximal increase in StAR protein was observed at 5–10 µg/ml hydroxysterol, while a further increase in StAR protein was seen with 15 µg/ml 25 OHC. Concentrations above 15 µg/ml were cytotoxic (data not shown). StAR protein levels were maximally increased over 24 h 6-fold by 20αOHC and 4-fold by 22(R)OHC and 25 OHC. Similarly, when MA-10 cells were treated for 6 h with 20αOHC, 22(R)OHC or 25 OHC, all three oxysterols caused a robust elevation of StAR protein levels, with 25 OHC being the most potent (Fig. 2A and B). Although the fold increase seen in oxysterol-treated MA-10 cells was greater than that seen in Y1 cells, this appears to be due at least in part to a higher basal level of StAR protein expression in Y1 cells. Consistent with previous results with Leydig cells (Lukyanenko *et al.* 2001), MA-10 cells were resistant to cytotoxicity by oxysterols.

When Y1 cells were incubated with either 20αOHC or 22(R)OHC for various time periods, we found that both oxysterols rapidly increased StAR protein within 1 h (Fig. 3). StAR levels continued to rise over 24 h with 20αOHC treatment, whereas levels remained relatively constant after 6 h with 22(R)OHC. Some effect was also seen with EtOH alone, but it was always substantially less than with oxysterols.

Various sterols and steroids increase StAR protein levels

To determine if other sterols and steroid products could regulate StAR protein levels, cells were incubated with free cholesterol, 7-ketocholesterol (7 ketoC), 21-hydroxypregnenolone (21 OHpreg), progesterone, pregnenolone, and dehydroepiandrosterone (DHEA) (Fig. 4A and B). We found that the oxysterol 7 ketoC also increased StAR protein, as did the steroids pregnenolone, progesterone and DHEA. In contrast, treatment with free cholesterol or 21 OHpreg had no effect on StAR protein expression.

Figure 1 Oxysterols increase StAR protein levels in Y1 adrenocortical cells in a concentration-dependent manner. Y1 cells were incubated with EtOH alone or varying concentrations of 20α-OHC, 22(R)OHC or 25 OHC for 24 h. All three oxysterols increased StAR protein expression as determined by immunoblot analysis. This experiment was repeated twice with duplicate samples.

Oxysterols increase StAR mRNA levels in steroidogenic cells

To ask if the oxysterol-mediated increase in StAR protein abundance correlates with an increase in mRNA, Northern blot analysis was performed on Y1 cells treated for 24 h with various oxysterols. As seen in Fig. 5A and B, StAR mRNA increased in response to treatment with 20αOHC and 22(R)OHC but not 25 OHC. The relative potency of the oxysterols was similar to that seen for StAR protein, with 20αOHC eliciting the greatest increase (Fig. 5B).

To determine if StAR mRNA levels correlated with the increase in StAR protein, the time course of induction of StAR mRNA in 20αOHC-treated Y1 cells was determined (Fig. 5C). Interestingly, mRNA levels increased considerably after StAR protein levels began to rise. Thus, while StAR protein increased within 1 h of oxysterol addition (Fig. 3), StAR mRNA levels did not increase until after 8–12 h.

Similarly, MA-10 cells were treated with 20αOHC, 22(R)OHC or 25 OHC for 6 or 24 h.

Northern blot analysis again revealed that short-term treatment had no effect on StAR mRNA levels (Fig. 6A and C). In contrast, after a 24 h incubation with oxysterol, StAR mRNA levels in MA-10 cells increased substantially over the control (Fig. 6B and C). This suggests that the acute increase in StAR protein abundance is not a result of increased transcription or mRNA stability. However, the persistent elevation in StAR protein could be due to the later rise in StAR mRNA levels.

To further explore whether the acute increase in StAR protein was due to increased stability or synthesis, Y1 cells were pretreated with the protein synthesis inhibitor cycloheximide prior to the addition of 20αOHC, and the accumulation of StAR protein over time was determined by immunoblot analysis (Fig. 7). As expected, treatment with cycloheximide significantly reduced the basal level of StAR protein in Y1 cells. Expression of StAR protein increased over time in cells treated with 20αOHC, but this increase was blocked by pretreatment with cycloheximide. Taken together, these data indicate that oxysterols acutely regulate StAR protein abundance at the level of translation.

Figure 2 Oxysterols increase StAR protein levels in MA-10 Leydig cells. (A) MA-10 cells were treated for 6 h without (Control) or with 1 mM dbcAMP (dbcA) or 15 µg/ml indicated oxysterol. Levels of StAR protein were significantly increased with 25 OHC, 20αOHC and 22(R)OHC as shown by immunoblot analysis. (B) Quantification of data from four similar experiments. The data are expressed as mean fold increase over control ± S.D for four experiments.

Oxysterol action is independent of StAR promoter activity

To determine if the chronic increase in StAR mRNA abundance was due to changes in transcription, Y1 cells were transiently transfected with a mouse *StAR* gene promoter reporter plasmid, p-966 StAR/luc. Treatment of transfected cells with either 20αOHC or 22(R)OHC for 24 h did not increase StAR promoter activity over levels in control or vehicle-treated cells (Fig. 8). Likewise, no increase in StAR promoter activity was observed when transfected cells were treated with oxysterols for 6–8 h (data not shown). In contrast, a known regulator of the StAR promoter, LDL, elicited a 2-fold increase in luciferase activity. Similar results were seen when Y1 cells were transfected with pGL1-3 kbStAR, which contains 1.3 kb of the human StAR promoter upstream of luciferase (data not shown). No significant effect of EtOH on mRNA levels or promoter activity was observed. These findings indicate that the oxysterol-stimulated elevation in StAR mRNA is independent of any change in the rate of *StAR* gene transcription.

Discussion

Steroidogenic cells rapidly respond to trophic hormone stimulation with increased expression of

Figure 3 Oxysterols increase StAR protein levels in Y1 adrenocortical cells in a time-dependent manner. Y1 cells were incubated with 15 µg/ml 20αOHC or 22(R)OHC or EtOH alone (Et) for different time periods (given in hours). Both oxysterols increased StAR protein levels as analyzed by immunoblot. This experiment was performed twice.

Figure 4 StAR protein levels are upregulated by both sterols and steroids. Y1 cells were incubated with 15 µg/ml free cholesterol (FC), 7 ketoC, 21 OHpreg, progesterone (Prog), pregnenolone (Preg), and DHEA or EtOH alone (lane marked C) for 24 h. (A) Representative immunoblot of StAR protein levels. (B) Quantification of data from five similar experiments. The data are expressed as fold increase over control $\pm$ S.D. (Cont). With the exception of FC and 21 OHpreg, all values are significantly different from the control ($P < 0.05$).

StAR and consequent steroid production. Given the essential role of StAR in steroid biosynthesis, much effort has been concentrated on understanding how this protein is regulated (Stocco *et al.* 2001). In addition to regulation at the transcriptional level, translational as well as post-translational control of StAR gene expression has been reported. The present data demonstrate that oxysterols, potent regulators of cholesterol and lipid metabolism (Björkhem 2002), may contribute to post-transcriptional regulation of StAR mRNA and protein.

Oxysterols exert a time-dependent biphasic effect on StAR expression in both Y1 and MA-10 cells. The early phase (0–6 h) appears to be independent of changes in StAR mRNA abundance, but requires new protein synthesis. This suggests that oxysterols may act to stabilize StAR protein or increase the rate of StAR mRNA translation. With prolonged stimulation (8–12 h), oxysterols also increase StAR mRNA levels, which may in turn

promote the continued rise in the amount of StAR protein. These data suggest that in addition to functioning as a substrate for StAR-independent steroidogenesis via direct conversion to steroid, oxysterols may regulate the production of steroids indirectly through post-transcriptional regulation of StAR activity. This is in agreement with previous studies that have shown that production of StAR protein and steroid in the initial moments of trophic hormone stimulation may be independent of changes in mRNA abundance. Similar observations have been made for basal, potassium-induced, and aspects of cAMP-stimulated StAR protein and steroid production (Clark *et al.* 1997, Clark & Combs 1999, Artemenko *et al.* 2001). Since no precursor pool of StAR exists in the cell and the accumulation of StAR is cycloheximide-sensitive, these data suggest that StAR is translationally regulated. However, evidence for changes in the rate of StAR translation, especially in the early moments of hormonal stimulation, remains elusive

Figure 5 Oxysterols increase StAR mRNA levels in Y1 cells in a time-dependent manner. Y1 cells were incubated with 15 μ g/ml 20 α OHC, 22(R)OHC or 25 OHC or ETOH alone (ETOH) for 24 h. StAR mRNA expression was then assessed by Northern blot (A, top panel). The blot was later stripped and re-probed for GAPDH mRNA to control for loading differences (bottom panel). (B) Results of Northern blot analyses were quantified and normalized to GAPDH expression. The data from four experiments were then averaged and are expressed as fold increase over control $\pm$ S.E.M. By Students' *t*-test, StAR mRNA levels were significantly increased by 20 α OHC and 22(R)OHC ($P < 0.05$) unlike with 25 OHC ($P = 0.08$). (C) Time course of StAR mRNA expression in cells treated with 20 α OHC compared with ETOH control (Cont) at 24 h. StAR mRNA expression was normalized to GAPDH levels. This experiment was repeated three times. Values represent the average of duplicate measurements in a single experiment.

(Artemenko *et al.* 2001, Clark *et al.* 2001). Alternatively, oxysterols may simply reduce the degradation rate of non-functional mature StAR protein.

Figure 6 Oxysterols increase StAR mRNA levels in MA-10 cells. MA-10 cells were treated without (Control) or with 1 mM dbcAMP (dbcA) or 15 μ g/ml 20 α OHC, 22(R)OHC or 25 OHC for 6 h (A) or 24 h (B). Oxysterols, especially 25 OHC, increased levels of StAR mRNA at both time points, although far less than dbcAMP, as verified by comparison with 18S rRNA levels as a loading control (below top panels). These results are quantified in (C).

The most potent sterol compounds were the side-chain oxygenated sterols 20 α OHC and 25 OHC, which elevated levels of StAR protein 4- to

Figure 7 Acute regulation of StAR protein by oxysterol is inhibited by cycloheximide (cyc). Y1 cells were treated with 20αOHC (15 μg/ml) for 1–8 h, or pretreated with 50 μg/ml cycloheximide for 30 min prior to the addition of 20αOHC. As a control, cells were treated with EtOH alone or cycloheximide alone for 2 or 8 h. StAR protein expression (see arrow) was analyzed by immunoblot as described. This experiment was performed twice with duplicate samples.

6-fold within 6 h. Interestingly, 20αOHC was most effective in increasing StAR protein in Y1 cells, while 25 OHC was more effective in MA-10 cells. Sterol oxidized in the 7 position also significantly stimulated StAR protein levels, whereas free cholesterol had no effect, possibly due to a low rate of uptake by the cells (Meaney *et al.* 2002). Steroids such as DHEA and pregnenolone also increased StAR protein levels, although generally not as strongly as oxysterols. This is consistent with previous data obtained with progesterone, where interestingly, StAR mRNA levels were found to be increased (Schwarzenbach *et al.* 2003). However, it is unclear whether progesterone, unlike oxysterols, increases StAR gene transcription since another report showed no effect of progesterone on promoter activity (Christenson *et al.* 1998). Interestingly, oxidation of carbon-21 in the pregnenolone side-chain eliminated its ability to upregulate StAR

Figure 8 Oxysterols do not increase StAR transcription. Y1 cells transiently transfected with p-966 StAR/luc and pCMVβ-galactosidase were incubated without (Cont) or with EtOH (ETOH), 15 μg/ml 20αOHC or 22(R)OHC or 50 μg/ml LDL for 22–24 h. Luciferase and β-galactosidase activities were assayed and values are expressed as relative light units (RLUs) of luciferase normalized to β-galactosidase activity. Values are the average of quadruplicate samples in the same experiment ± s.d., and are representative of four similar experiments, two for LDL.

protein. *In toto*, these results point to a lack of feedback inhibition of steroidogenesis *in vitro*. In support of this, chronic trophic stimulation of steroidogenic cells causes excessive StAR mRNA and protein levels to subside only partially from their peak (Clark *et al.* 1995). Thus, the apparent decline in steroid output observed with chronic hormone stimulation may primarily result from increased steroid metabolism and the suppression of enzymes downstream in the steroidogenic pathway, with a resulting increased synthesis of alternative steroids (Yee & Hutson 1985, Rommerts *et al.* 2001, Chen *et al.* 2002, Javitt 2002).

While oxysterol treatment did not cause an acute increase in StAR mRNA, the later increase in mRNA levels was surprising, differing from previous observations in granulosa cells incubated in delipidated media, which might still contain serum oxysterol (Christenson *et al.* 1998). Interestingly, although transcriptional regulation by oxysterols has been debated, we found no consistent effect on transcription from either the mouse or human StAR promoter at any of the time points examined (Lala *et al.* 1997, Christenson *et al.* 1998, Mellon & Bair 1998). It may be that the relevant binding element for oxysterol action was excluded in the promoter fragments utilized, or that the later increase in StAR mRNA levels simply reflects a lowered degradation rate, as previously observed with actinomycin D (Clark *et al.* 1997).

In contrast to oxysterols, LDL increases StAR expression through upregulation of StAR promoter activity using an SF-1 site at –135 in the murine StAR promoter, suggesting involvement of SF-1 (Reyland *et al.* 2000). The effect of LDL would probably not be enhanced by oxysterols since neither the P450 scc inhibitor aminoglutethimide, nor ketoconazole, which inhibits synthesis and

conversion of cholesterol to hydroxycholesterol or steroid, affects LDL-induced increases in StAR mRNA levels (Reyland *et al.* 2000). Taken together, these data suggest that increased substrate cholesterol availability may promote StAR protein synthesis. However, oxysterols and lipoprotein act via distinct mechanisms, with LDL acting primarily at the level of transcription while oxysterols appear to act post-transcriptionally.

The importance of oxysterol regulation of StAR is unclear, given how minor its effect is compared with dbcAMP. Oxysterol action may be essential for increasing steroid synthesis in the first minute of trophic hormone stimulation through translational regulation or stabilization of the active form of StAR (Clark & Combs 1999, Artemenko *et al.* 2001, Clark *et al.* 2001). The level of steroid consequently elicited may be appropriately small in order to be non-depleting, since cholesterol supply pathways are not activated by oxysterol (Javitt 2002). This would be advantageous since low steroid hormone concentrations may permit more targeted, local actions by steroid hormones as opposed to the systemic effects mediated by high endocrine levels generated in response to pituitary signaling. The higher sensitivity of MA-10 cells to 25 OHC may reflect just such a physiological situation in the testis. There, comparable levels of 25 OHC produced by testicular macrophages can induce differentiation of primary rat progenitor and immature Leydig cells and long-lasting increases in 'basal' steroidogenesis in adult Leydig cells; 25 OHC further elicits the generation of up to 3-fold higher concentrations of testosterone than of the oxysterol itself, indicating that the induced production of steroid is not simply a result of direct conversion of 25 OHC to testosterone (Yee & Hutson 1985, Lukyanenko *et al.* 2001, Chen *et al.* 2002). Thus, the loss of oxysterol production in testes depleted of macrophages may account for the observed reduction in steroidogenic enzyme and testosterone levels (Bergh *et al.* 1993, Cohen *et al.* 1997).

The present data are consistent with a general role for oxysterols in cholesterol metabolism (Javitt 2002). Oxysterols upregulate pro-lipogenic SREBP-1c activity and suppress *de novo* cholesterol biosynthesis possibly by inhibiting SREBP-1a activation, even under conditions of cholesterol deprivation in steroidogenic cells (Schuler *et al.* 1979, Rennert *et al.* 1990, Lehmann *et al.* 1997,

Repa *et al.* 2000, Christenson *et al.* 2001, Fu *et al.* 2001, Björkhem 2002, Horton *et al.* 2002). Interestingly, SREBP-1a, whose activity would be predicted to rise with prolonged steroidogenesis, may increase StAR synthesis (Christenson *et al.* 1998, 2001, Shea-Eaton *et al.* 2001). However, inhibition of endogenous SREBP-1a by 27 OHC and LDL does not alter StAR promoter activity (Christenson *et al.* 2001). Thus, the apparent effect on *StAR* gene expression might normally be mediated by SREBP-1c or indirectly through SREBP-1a-induced increases in cholesterol. This may help explain why rat R2C Leydig tumor cells which contain high levels of free cholesterol constitutively synthesize StAR and steroid (Rao *et al.* 2003).

In addition to regulation of StAR, oxysterols can be directly converted to steroid. This process may help explain how small amounts of steroid can still be made in *StAR*^{-/-} mice and in the human placenta, which lacks StAR (Caron *et al.* 1997b, Pollack *et al.* 1997). When highly purified cytotrophoblasts are cultured in the absence of serum, steroid production appears to cease after 1 h, indicating a loss of stimulus or an exhaustion of substrate, such as serum-derived oxysterol (Kliman *et al.* 1986). Given the general availability of oxysterols from peripheral, local and intracellular sources, we propose that these compounds may be responsible for much of the observed StAR-independent steroidogenesis. The remainder may be a result of the unidentified factor that stimulates mitochondrial 27-hydroxylase activity in the alternative pathway of bile acid synthesis (Vlahcevic *et al.* 1994). These two effects of oxysterols on StAR and steroidogenesis may serve to regulate cell function locally, such as in the testis through 25 OHC, and the brain, which produces significant levels of 24 OHC (Lutjohann *et al.* 1996, King *et al.* 2002).

In summary, oxysterols increased both StAR protein and mRNA levels in a dose- and time-dependent manner in adrenocortical and Leydig cell lines with no observed effect on promoter activity. Furthermore, protein levels increased prior to detectable changes in mRNA were dependent upon new protein synthesis. Thus, we conclude that in the short term, oxysterols increase StAR protein levels independently of increased gene transcription and mRNA abundance, perhaps a result of translational regu-

lation. We propose that oxysterols contribute to steroidogenesis through modulation of StAR activity and the direct conversion to steroid, which probably accounts for a significant proportion of StAR-independent steroid production in the body.

Acknowledgements

We thank Dr William K Wilson (Rice University, Houston, TX, USA) for critical reading of the manuscript. This work was supported by grants DK50690 (to M E R), DK61548 (to S R K) and HD17481 (to D M S) from the National Institutes of Health as well as funds from the Robert A Welch Foundation (to D M S) and the Lalor Foundation (to S R K).

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Received in final form 18 November 2003

Accepted 30 November 2003