Transcription of *LIPE* gene encoding hormone-sensitive lipase/cholesteryl esterase is regulated by SF-1 in human adrenocortical cells: involvement of protein kinase A signal transduction pathway

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Abstract

The study was designed to elucidate the influence of the protein kinase A (PKA) signal transduction pathway on transcription of the *LIPE* gene encoding hormone-sensitive lipase/cholesteryl esterase (HSL) in H295R cells. HSL is one of the key enzymes involved in steroid hormone synthesis, and ACTH, with mediation of the PKA pathway, increases its activity. However, the mode of regulation of *LIPE* gene expression by ACTH remains unknown. It was found that stimulation of the PKA pathway by the adenylyl cyclase activator, forskolin, caused a twofold increase in *LIPE* transcript accompanied by appreciable rise in the protein product of the gene and cortisol output. RNA polymerase II inhibitor abolished, and protein synthesis inhibitor attenuated this effect. Forskolin and PKA catalytic subunit increased transcriptional activity of *LIPE* promoter A in cells transfected with the luciferase reporter vector. Overexpression of steroidogenic factor-1 (*SF-1*) increased *LIPE* promoter activity, while transient silencing of *SF-1* expression with specific siRNAs abolished forskolin-stimulated *LIPE* transcription. It is concluded that ACTH via the PKA pathway stimulates expression of *SF-1*, which activates transcription of *LIPE* presumably by interaction with putative binding sequences within promoter A. A novel mechanism contributing to the long-term effect of ACTH on adrenal steroidogenesis is proposed: ACTH stimulates transcription of *SF-1*, which interacts with the putative SF-1-binding sequences within the promoter and activates *LIPE* transcription. An increased level of HSL results in an enhanced supply of cholesterol required for steroid hormone synthesis.

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Introduction

Steroid hormone synthesis in the adrenal cortex requires a continuous supply of free cholesterol. However, endogenous synthesis provides only about 20% of the cholesterol required for corticosteroid formation (Kraemer 2007), the main source being plasma lipoproteins, low-density lipoprotein (LDL) and high-density lipoprotein (HDL) (Azhar & Reaven 2002, Connelly & Williams 2004). LDL is taken up by the cells via LDL receptor-mediated endocytosis, and free cholesterol is liberated from cholesteryl esters in lysosomes by ‘acid’ cholesteryl esterase (EC 3.1.1.13). The uptake of HDL cholesterol requires scavenger receptor class B type 1 (SR-B1) and is limited to cholesteryl esters, which are hydrolysed in the cytosol by hormone-sensitive lipase/cholesteryl esterase (HSL) EC 3.1.1.79 (Rodriguez *et al.* 1999). In the cytosol, free cholesterol is esterified with activated fatty acids by acyl-CoA cholesterol acyl transferase (ACAT)

(EC 2.3.1.26). Cholesteryl esters formed are stored in lipid droplets, which mainly contain cholesteryl esters and phospholipids with some proteins and negligible amounts of free cholesterol and triacylglycerols (Boyd & Trzeciak 1973).

The main source of substrate for steroid hormone synthesis is free cholesterol liberated by HSL from cholesteryl esters stored in lipid droplets (Trzeciak & Boyd 1973). HSL is a fatty acyl hydrolase that cleaves fatty acyl esters of glycerol and cholesterol (Kraemer & Shen 2002). This enzyme plays an important role in the control of energy homeostasis by releasing free fatty acids from acylglycerols stored in adipocytes for transport and oxidation in energy-requiring tissues (Yeaman 1990). HSL also regulates provision of free cholesterol for steroid hormone synthesis in the adrenal cortex, testis, ovary and placenta. HSL is most active in adipose tissue. In steroidogenic tissues including the adrenal cortex, the enzyme is about three times less active, and its activity in pancreatic

β -cells is even lower, while in the heart and macrophages, it is negligible (Khoo *et al.* 1993, Langfort *et al.* 1999, Lindvall *et al.* 2004).

It is well known that both cholesterol release and the amount of steroid hormones produced in the adrenal cortex are regulated by ACTH (Trzeciak & Boyd 1974, Boyd *et al.* 1975). ACTH binds to a specific receptor coupled with membrane-bound adenylyl cyclase; thus, cAMP is generated and activates the protein kinase A (PKA) signal transduction pathway, which leads to phosphorylation and activation of HSL (Trzeciak & Boyd 1974).

With the exception of the catalytic site, the primary structure of HSL differs from that of other lipases. Within the structure of HSL, two functional domains can be distinguished. The N-terminal domain is responsible for interaction with lipase translocating protein (lipotransin), adipose lipid-binding protein (ALBP) and steroidogenic acute regulator (StAR; Shen *et al.* 1999, 2003, Syu & Saltiel 1999). The C-terminal domain comprises the substrate-binding site, catalytic triad: Ser⁴²⁴, Asp⁶⁹² and His⁷²³, and the regulatory loop containing several Ser residues which can be phosphorylated by different kinases (Contreras *et al.* 1996). A particular role is played by Ser⁵⁶³ which is phosphorylated by PKA (Holm 2003). Following PKA-dependent phosphorylation of Ser⁵⁶³, HSL forms a complex with lipotransin which translocates the enzyme to the surface of lipid droplets (Egan *et al.* 1992, Syu & Saltiel 1999). Owing to the ATPase activity of lipotransin and at the expense of a high-energy bond of ATP, HSL is liberated from the complex with lipotransin in order to exert its catalytic function (Syu & Saltiel 1999). Activation of PKA also results in phosphorylation of perilipins, covering lipid droplets and preventing access of HSL to its substrate (Londos *et al.* 1995, Servetnick *et al.* 1995). Upon phosphorylation, perilipins dissociate, and HSL is capable of binding to the surface of lipid droplets and hydrolysing cholesteryl esters. All effects mentioned above are parts of the short-term regulation of steroidogenesis by ACTH and precede the long-term regulation involving stimulation of transcription of genes encoding steroidogenic cytochromes P450 (*CYP*), 3- β -hydroxysteroid dehydrogenase (*HSD3B*) and *StAR* (Orth & Kovacs 1998).

In the adipocyte line 3T3-F442A, stimulation of the PKA pathway results in inhibition of transcription of the *LIPE* gene, encoding HSL (Plee-Gautier *et al.* 1996). On the contrary, in Leydig cells, human chorionic gonadotrophin acting through the PKA pathway induces expression of this gene (Kraemer *et al.* 1993).

To date, however, it has not been shown whether activation of the PKA pathway is accompanied by increased transcription of *LIPE* in adrenocortical cells.

Such finding might constitute a novel, important element in the long-term response to ACTH.

The *LIPE* gene, located on chromosome 19q13.1, is composed of nine exons plus six additional exons (A, B, C, D, T1 and T2), which are transcribed in different tissues, and the transcription is controlled by tissue-specific promoters (Levitt *et al.* 1995, Laurin *et al.* 2000).

In adipose tissue, expression of *LIPE* is controlled by promoter B located upstream from non-coding exon B and containing exon 1. In the adrenal cortex, however, expression of this gene is driven by promoter A, situated 15 kb upstream from exon 1 (Grober *et al.* 1997). Promoter A precedes exon A which encodes a translation start codon, and this extends the open reading frame and results in the addition of 43 amino acids to the N-terminal end of HSL. In the testes, promoter T, located more than 15 kb upstream of exon 1, contains two additional exons, T1 and T2. Exon T1 encodes a translation start codon and additional 301 amino acids of the N-terminus of HSL, while transcription of exon T2 results only in extending the 5'-UTR of the transcript (Blaise *et al.* 1999). Like exon T2, non-coding exons C and D extend the 5'-UTR of the transcripts. The presence of different promoters results in the differential expression of three isoforms of human HSL. In adipocytes, it is mostly the 84 kDa form which is expressed, and in adrenal cortex and pancreatic β -cells, the 89 kDa form is the major one, whereas in the testes, the 120 kDa form predominates.

To date, investigations have been focused on regulation of the *LIPE* expression in adipose tissue, where the 84 kDa isoform predominates. The main regulators of *LIPE* expression in the adipose tissue are glucose and glucocorticoids (Slavin *et al.* 1994, Botion & Green 1999, Smih *et al.* 2002). However, in the adrenal cortex, the expression of the 89 kDa isoform has not been investigated. Elucidation of the ACTH effect on transcription of *LIPE* gene, in particular the activity of promoter A, is therefore essential for understanding mechanisms regulating substrate supply for steroid hormone synthesis in the adrenal cortex.

It has been firmly established that steroidogenic factor-1 (SF-1) is involved in the regulation of steroidogenic gene expression by ACTH (Morohashi *et al.* 1992, Val *et al.* 2003). SF-1 binds to specific binding sequences in target gene promoters and regulates transcription of a number of genes including *CYP*, *HSD3B* and *StAR*. SF-1 also plays an important role in the differentiation of cells producing steroid hormones.

This study has been designed to determine whether transcription of the *LIPE* gene encoding HSL is regulated by ACTH via cAMP and the PKA signal transduction pathway in human adrenocortical cells.

An attempt has also been made to identify transcription factor(s) responsible for *LIPE* expression and their target sequences in *LIPE* promoter A.

Materials and methods

Materials

Forskolin, 8Br-cAMP, 5,6-dichloro-1- β -D-ribofuranosylbenzimidazole (DRB), cycloheximide (CX), insulin, transferrin, sodium selenite mixture (ITS) and antibiotic/antimycotic (ABAM) were purchased from Sigma–Aldrich. Foetal bovine serum (FBS) was obtained from Gibco, and Ultrosor G was a product of Pall Corporation (Port Washington, NY, USA).

TRIityd G reagent was purchased from Applichem (Darmstadt, Germany); reverse transcription kit and random hexamers were purchased from Novazym (Poznan, Poland); LC FastStart DNA Master SYBR Green1 and FuGene HD were purchased from Roche Diagnostics and Dual-Luciferase Reporter Assay System was purchased from Promega.

Antibody directed against HSL was obtained from Cell Signalling Technology, Inc. (Danvers, MA, USA), while anti-SF-1 antibody, anti- β -actin antibody and SF-1 siRNA were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Protein Assay Dye reagent was obtained from Bio-Rad, and the Western Blotting Detection System was supplied by Amersham.

Constructs

The pGL3 vector, containing the *Firefly* luciferase gene under the control of *LIPE* promoter A fragments, was donated by Dr C Holm (Lund, Sweden), and the reference vector, harbouring *Renilla reniformis* gene (pRL-TK), was supplied by Dr J Li (Lyon, France). Expression vector pCHV-SF-1 harbouring the *SF-1* gene was presented by Dr K Parker (Dallas, USA), and the pMT-CMV α vector, containing cDNA of the α subunit of PKA, was donated by Dr G S McKnight (Washington DC, USA).

Cell culture and treatments

Human adrenocortical cells (line H295R), purchased from American Type Culture Collection (Manassas, VA, USA), were cultured in combined DMEM/Ham's F-12 1:1 (v/v) containing L-glutamine (2.5 mM), which was obtained from Sigma–Aldrich. The medium was supplemented with 3% FBS, 4% Ultrosor G, 1% ITS (1.0 mg/ml insulin, 0.55 mg/ml transferrin and 0.5 μ g/ml sodium selenite) and ABAM. Other reagents used for cell culture were purchased from Gibco. Cortisol concentration in the culture medium was estimated by electrochemiluminescence using the Cobas 6000 instrument (Roche Diagnostics).

Incubation of the H295R cells with test substances

After confluence was reached, 24 h before treatment, the medium was substituted with the same medium but it was serum-free, containing ITS and test substances: forskolin, DRB or CX. Incubation was conducted for either 24 or 48 h as indicated. All incubations with test substances were conducted in serum-free medium. After incubation, the cells were washed with PBS, harvested and lysed with modified radioimmunoprecipitation assay buffer.

Isolation of RNA, reverse transcription and amplification of cDNA

RNA was isolated according to the phenol–chloroform method (Chomczynski & Sacchi 1987) using TRIityd reagent (Applichem). The concentration of RNA was measured by spectrophotometry (Eppendorf, Hamburg, Germany), and 1 μ g of RNA was transcribed with the use of MMLV reverse transcriptase and random hexamers (Novazym) according to the manufacturer's instructions.

Quantitative analysis of the *LIPE* and *SF-1* transcripts was conducted by real-time PCR (RT-qPCR) with the use of the LightCycler1.0 System (Roche Diagnostics) and specific primers (Table 1). Results were normalised to the mitochondrial ribosomal protein L19 gene (*MRPL19*), which was used as the reference.

Table 1 Oligonucleotide primers used for RT-qPCR

cDNA	Primers forward (F) and reverse (R)	Annealing temperature (°C)	Product size (bp)
<i>LIPE</i>	F: 5'-GCGGTGGCGAAAAGACAAG-3' R: 5'-GGTCCAGGTCAAAGAGGTG-3'	62	239
<i>SF-1</i>	F: 5'-ACCCACAGTCGCCACCGT-3' R: 5'-GAGCAGTCCGTAGTGGTAG-3'	60	201
<i>MRPL19</i>	F: 5'-ACTTTATAATCCTCGGGTC-3' R: 5'-ACTTTCAGCTCATTAACAG-3'	62	171

Incubation of the cells with the luciferase reporter vectors and determination of luciferase activity

The cells were transfected with the reporter vector (pGL3) harbouring the *Firefly* luciferase gene under control of the *LIPE* promoter A fragments (–30, –60, –343, –1150 and –2150 bp) together with the expression vector harbouring *SF-1* gene or pMT-CEV α vector containing the *PRKACA* gene encoding catalytic subunit α ($C\alpha$) of PKA. For transfections, FuGene HD reagent (Roche Diagnostics) was used. After transfection, the cells were incubated for 48 h, harvested and lysed. *Firefly* luciferase activity in the cell lysates was determined using the Dual Luciferase System and 20/20n Luminometer (Turner BioSystems, Sunnyvale, CA, USA). Transfection efficiency was normalised to the co-transfected reference vector pRL-TK containing the *Renilla* luciferase gene.

Transfection of the cells containing *SF-1* siRNAs and determination of *LIPE* expression level

Silencing of *SF-1* expression was achieved by transfection of H295R cells with the mixture of three siRNAs complementary to the *SF-1* transcript (Santa Cruz). Transfection was conducted under optimal conditions, and the FuGene HD reagent was used. The effectiveness of silencing was monitored by western blotting at 24 and 48 h after transfection. *SF-1* levels were estimated using anti-*SF-1* antibody followed by HRP-conjugated anti- γ -globulin secondary antibody and detection of peroxidase activity. The effect of *SF-1* silencing on the level of *LIPE* transcript was determined by RT-qPCR, while the influence of *SF-1* silencing on HSL protein level was determined by western blotting with the use of specific antibody and HRP-conjugated secondary antibody.

Computer software and Internet addresses

Primers were designed using Oligo v.6.71 software, and DNA sequences were obtained from GenBank (<http://www.ncbi.nlm.nih.gov/BLAST/>), Genew: human gene nomenclature Database <http://www.gene.ucl.ac.uk/egi-bin/nomenclature/>).

Statistical analysis

Statistical analysis of the results was carried out with the aid of GraphPad InStat v.3.05 (GraphPad Software, La Jolla, CA, USA) and Microsoft Excel 2007. The results are the mean ($\pm$ S.E.M.) from at least two duplicates from three (or more) separate experiments. To estimate the influence of test substances on the level of transcripts, one-way ANOVA or two-way ANOVA tests were applied. All results were also tested with *post-hoc*

Student–Newman–Keuls test. Significance of the differences between individual samples was tested at the level of $*P < 0.05$, $**P < 0.01$ or $***P < 0.001$.

Results

Stimulation of the PKA pathway results in induction of *LIPE* transcription

It is known that ACTH, acting via the PKA pathway, induces steroidogenic gene expression in the adrenal cortex. Therefore, we assumed that *LIPE* transcription might also be affected. Since H295R cells lack ACTH receptor, we stimulated the PKA pathway with the adenylyl cyclase activator, forskolin, or alternatively with 8Br-cAMP. Upon stimulation of the PKA pathway for 24 h, a two- to threefold increase in the level of the *LIPE* transcript, accompanied by appreciable increase in the protein product of the gene and cortisol output, was observed (Fig. 1). Although after incubation for 36 or 48 h without or with forskolin the level of the *LIPE* transcript was about 20% higher than after 24 h (not shown), the twofold increase in the transcript level was insignificant. An inhibitor of RNA polymerase II, DRB, abolished this effect, indicating that stimulation of the *LIPE* expression took place at the transcriptional level.

In order to examine whether stimulation of *LIPE* expression depends on the synthesis of proteins, which might affect transcription of the gene, we incubated the cells with forskolin and/or protein synthesis inhibitor, CX. It was found that CX slightly lowered the basal expression level and significantly alleviated the effect of forskolin on *LIPE* transcription (Fig. 1), suggesting that

Figure 1 Effects of forskolin, 8Br-cAMP and an inhibitor of RNA polymerase II or protein synthesis inhibitor on the level of the *LIPE* transcript. The H295R cells were incubated for 24 h without (control) or with 25 μ M forskolin, 0.1 μ M 8Br-cAMP and/or 75 μ M 5,6-dichloro-1- β -D-ribofuranosylbenzimidazole (DRB) or else with 10 μ g/ml cycloheximide (CX). Following incubation, total RNA was extracted, and cDNA was obtained by reverse transcription. Specific cDNA was then amplified and quantified by PCR (RT-qPCR). The results are the average of four separate experiments $\pm$ S.E.M.; $***P < 0.001$; $**P < 0.01$. The HSL level, determined by western blotting (fold control is shown in brackets), and cortisol output without forskolin (C) and under forskolin stimulation (F) are shown in the insets.

newly synthesised protein(s) are required for stimulation of the *LIPE* transcription.

The state of chromatin condensation, which also might be affected by stimulation of the PKA pathway, was excluded since histone deacetylase inhibitor, trichostatin A, did not influence the stimulatory effect of forskolin on the *LIPE* expression (not shown).

The mode of regulation of transcriptional activity of *LIPE* promoter A by PKA

To investigate the regulation of transcriptional activity of promoter A by the PKA pathway, H295R cells were transfected with the vector containing *Firefly* luciferase gene under control of the –2150 bp fragment of the *LIPE* promoter and were incubated with forskolin, which significantly increased its transcriptional activity. This suggested that stimulation of the PKA pathway increased the expression of *LIPE*. As expected, in the cells transfected with the luciferase reporter vector containing the same promoter fragment cloned in an inverted position, very low transcriptional activity and no effect of forskolin were observed (Fig. 2). Overexpression of the catalytic subunit α of PKA (C α) caused a greater than twofold increase in the transcriptional activity of promoter A (Fig. 2) documenting that the effect of forskolin is mediated by PKA.

The PKA pathway regulates transcription of the SF-1 gene

SF-1 is the principal transcription factor involved in the expression of a number of steroidogenic genes whose transcription is controlled by the PKA pathway (Morohashi *et al.* 1992, Val *et al.* 2003). Therefore, we presumed that stimulation of the PKA pathway might also affect *SF-1* transcription and decided to evaluate the effect of forskolin on the level of *SF-1* transcript.

Figure 2 Transcriptional activity of the *LIPE* promoter A as affected by forskolin or catalytic subunit of protein kinase A. The H295R cells were transfected with the pGI3 vector containing a –2150 fragment of the *LIPE* promoter A in normal or inverted position (inv) directing *Firefly* luciferase reporter gene expression. The cells were either incubated for 48 h without (control) or with 25 μ M forskolin or were co-transfected with the vector expressing catalytic subunit α of protein kinase A (C α) and were incubated for 48 h. After incubations, luciferase activity was determined, as described in the Materials and methods section. RLU, relative luciferase units. The results are the average of four separate experiments $\pm$ S.E.M.; ** P < 0.01.

Figure 3 The influence of forskolin on *SF-1* transcript levels. The H295R cells were incubated for 24 h without (control) or with 25 μ M forskolin. Following incubation, total RNA was extracted. Specific cDNAs were then amplified and quantified by PCR (RT-qPCR). The results are the average of four separate experiments $\pm$ S.E.M.; *** P < 0.001. The *SF-1* level, determined by western blotting (fold control is shown in brackets), is shown in the inset.

As a result of 24 h incubation of H295R cells with forskolin, a greater than twofold increase in the level of *SF-1* transcript was observed (Fig. 3). This was accompanied by more than threefold increase in SF-1 protein (Fig. 3 inset). This suggested that the protein product of this gene might be involved in the induction of *LIPE* expression.

SF-1 stimulates transcriptional activity of *LIPE* promoter A

In order to demonstrate the effect of SF-1 on the activity of promoter A, the cells transfected with the luciferase reporter vectors under control of the promoter fragments ranging from –30 to –2150 bp were co-transfected with the vector expressing *SF-1*, and the luciferase activity was determined. It was shown that transfection of the same vector into COS cells, in which *SF-1* is not expressed, caused accumulation of SF-1 protein detected by western blotting with the use of specific antibody (not shown).

It was found that overexpression of SF-1 in H295R cells resulted in a more than twofold increase in the transcriptional activity of the –2150 and –1150 bp but not the shorter (–343, –100, –60 and –30) fragments of the promoter (Fig. 4). This indicates that SF-1 stimulates the expression of *LIPE* by interaction with the longer fragments of the promoter. Computer analysis of the DNA sequence of promoter A revealed two putative SF-1-binding sequences localised within the –1400 to –1420 bp region, suggesting direct interaction of SF-1 with the –2150 bp promoter fragment. The interaction of SF-1 with the –1150 bp fragment, which does not contain any SF-1-binding sequences, may be indirect and requires additional protein(s).

In order to demonstrate that SF-1 is essential for the PKA-mediated *LIPE* expression, H295R cells were

Figure 4 Influence of overexpression of *SF-1* on the transcriptional activity of the *LIPE* promoter A. The H295R cells were transfected with the pG13 vector containing deletion fragments of *LIPE* promoter A directing *Firefly* luciferase reporter gene expression (white bars). Parallel samples were co-transfected with the expression vector encoding *SF-1* (black bars). The cells were incubated for 48 h, and luciferase activity was measured as described in the Materials and methods section. RLU, relative luciferase units. The results are the average of four separate experiments $\pm$ s.e.m.; ** $P < 0.01$; * $P < 0.05$.

transfected with siRNAs complementary to the *SF-1* transcript. Transient silencing of the *SF-1* expression resulted in a major decrease in *SF-1* protein, while β -actin level was unaffected. The silencing of *SF-1* also caused a significant inhibition of forskolin-stimulated *LIPE* expression and a slight, but insignificant, attenuation of its basal expression (Fig. 5). This clearly shows that *SF-1* expression is required for stimulation of *LIPE* transcription by the PKA pathway. Silencing of *SF-1* did not affect basal HSL protein level; forskolin increased HSL protein content, while *SF-1* siRNA attenuated the effect of forskolin (Fig. 5 inset). Similarly, cortisol output was stimulated by forskolin, basal output was slightly lowered by *SF-1* siRNA, while *SF-1* silencing predictably did not influence the effect of forskolin since forskolin activates the PKA pathway, which results in phosphorylation and activation of HSL (not shown).

Discussion

To investigate *LIPE* transcription, we chose the human adrenal tumour cell line H295R, which has been widely used as a model system (Rainey *et al.* 1993, 1994, Staels *et al.* 1993). These cells contain all major steroidogenic enzymes, including HSL, and respond to stimulation of the PKA pathway by increasing steroid hormone output, but they lack the ACTH receptor. Therefore, to investigate the effects of stimulation of the PKA pathway, adenylyl cyclase activator (forskolin) or a cAMP derivative (8Br-cAMP) capable of crossing the cell membrane was used instead.

Stimulation of the PKA pathway by forskolin caused a greater than twofold increase in the amount of the *LIPE* transcript, quantified by real-time PCR. This was accompanied by an increase in the protein product of the gene and cortisol output. A similar result was obtained when 8Br-cAMP was applied. Since DRB, an inhibitor of RNA polymerase II, abolished the effect of forskolin, we concluded that an increase of *LIPE* expression took place at a transcriptional level. The inhibitory effect of CX, on the other hand, indicated that stimulation of *LIPE* transcription depended on *de novo* synthesis of an unknown protein(s), which might affect expression of this gene.

Moreover, forskolin significantly increased transcriptional activity of the *LIPE* promoter fragment controlling expression of the *luc* reporter gene. The effect was specific since it was achieved only when the promoter was cloned into the vector in the proper orientation.

A similar effect was obtained when cells were transfected with the reporter vector containing *LIPE* promoter together with the vector expressing the catalytic subunit α of PKA, demonstrating that PKA activity is required for the stimulation of *LIPE* transcription.

Taken together, these observations indicate that ACTH stimulates *LIPE* expression at a transcriptional level via cAMP and PKA and that a protein(s) synthesised *de novo* is (are) required for this stimulation.

During the search for a stimulatory protein, CREB was excluded since the *LIPE* promoter does not contain a cAMP-response element (CRE) (Smale 1997, Lindvall *et al.* 2004). The best candidate for such a protein was *SF-1*, a principal stimulator of expression of almost all genes involved in steroid hormone synthesis (Val *et al.* 2003). We found that stimulation of the PKA pathway led to a significant increase in the expression of *SF-1*.

Figure 5 Effect of forskolin on the *LIPE* expression in the cells in which *SF-1* is silenced. The cells were either left intact (C) or *SF-1* was silenced by introducing *SF-1* siRNAs. The cells were incubated without or with 25 μ M forskolin. After incubation, total RNA was extracted; cDNA was obtained by reverse transcription and was quantified by PCR (black bars). The results are the average of four separate experiments $\pm$ s.e.m.; *** $P < 0.001$. *SF-1* and β -actin protein content in the cells transfected with *SF-1* siRNAs is shown in the inset. In parallel experiments, the level of HSL protein was estimated in these cells by western blotting (inset) and quantified by densitometry (white bars).

Figure 6 Hypothetical mechanism of regulation of the *LIPE* transcription by the protein kinase A pathway in the H295R cells. AC, adenylyl cyclase; PKA, protein kinase A; SF-1, steroidogenic factor-1; HSL, hormone-sensitive lipase/cholesterol esterase. Asterisks denote activated forms.

This suggested that in the adrenal cortex, this pathway activated the expression of *SF-1*, thus supplying enough SF-1 protein required for stimulation of *LIPE* transcription.

In order to demonstrate the effect of SF-1 on *LIPE* transcription, we overexpressed *SF-1* in H295R cells transfected with the vector containing *luc* reporter gene under control of *LIPE* promoter fragments. This caused a greater than twofold increase in transcriptional activity of the -2150 and -1150 bp fragments but not the shorter ones. Analysis of the *LIPE* promoter sequence reveals the presence of two putative SF-1-binding sequences localised between -1400 and -1420 bp.

Transfection experiments revealed that *SF-1* overexpression also led to an increase in the transcriptional activity of the -1150 bp fragment of *LIPE* promoter. However, no SF-1-binding sequences were found in the entire fragment. Since activation of both *LIPE* promoter fragments by overexpression of SF-1 was almost equally strong, we postulate that the shorter fragment was activated by SF-1 indirectly and that other, yet unidentified, protein(s) was (were) required for this effect. We demonstrated that CX attenuated forskolin-induced transcription of *LIPE*. This suggested that a protein or proteins, synthesised *de novo*, were required for this effect. We postulate that one of those proteins might be SF-1.

Silencing of *SF-1* expression by specific siRNAs abolished the stimulatory effect of forskolin on *LIPE* expression. This indicates that in transduction of the

tropic signal from ACTH through its receptor, cAMP and PKA, SF-1 might play a role as the final regulator that directly or indirectly stimulates *LIPE* expression.

We conclude that ACTH stimulates expression of *LIPE* via the PKA signal transduction pathway, the principal regulator being the protein product of *SF-1*, and that the PKA pathway induces expression of this gene. Thus, we demonstrate the contribution of the *LIPE* and *SF-1* expression to the long-term effect of ACTH on steroid hormone synthesis in the adrenal cortex.

Our results enable us to propose a hypothetical mechanism by which the *LIPE* transcription is regulated in the adrenal cortex (Fig. 6). Stimulation of the PKA pathway by ACTH leads to an increase in the expression of *SF-1* and its protein product, and SF-1 by direct or indirect interaction with *LIPE* promoter A activates *LIPE* expression. Although our preliminary observations suggest that SF-1 might directly interact with SF-1-binding sequences localised in promoter A between -1400 and -1420 , SF-1 might also contribute to the stimulation of *LIPE* transcription by indirect interaction with the promoter region -1150 to -343 , which does not contain SF-1-binding sequences, but this would require an additional, yet unknown protein(s).

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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