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CHOLESTEROGENIC LANOSTEROL 14α-DEMETHYLASE (*CYP51)* IS AN IMMEDIATE EARLY RESPONSE GENE

Running title: Immediate early response of *CYP51* to cAMP stimuli

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Abstract

Lanosterol 14α-demethylase (CYP51) responds to cholesterol feedback regulation through sterol regulatory element binding proteins (SREBPs). The proximal promoter of *CYP51* contains a conserved region with clustered regulatory elements: GC box, cAMP-response elements (CRE-like) and sterol regulatory element (SRE). In lipid-rich (SREBP-poor) conditions the CYP51 mRNA drops gradually, the promoter activity is diminished and no DNA-protein complex is observed at the CYP51-SRE1 site. The majority of cAMP-dependent transactivation is mediated through a single CRE (*CYP51*-CRE2). Exposure of JEG-3 cells to forskolin, a mediator of the cAMP-dependent signaling pathway, provokes an immediate early response of *CYP51*, which has not been described before for any cholesterogenic gene. The CYP51 mRNA increases up to 4-fold in 2 hours and drops to basal level after 4 hours. The inducible cAMP early repressor ICER is involved in attenuation of transcription. Overexpressed CREB/CREM transactivate the mouse/human CYP51 promoters containing *CYP51*-CRE2 independently of SREBPs and ICER decreases the CREB-induced transcription. Besides the increased CYP51 mRNA, forskolin affects the *de novo* sterol biosynthesis in JEG-3 cells. An increased consumption of lanosterol, a substrate of CYP51, is observed together with modulation of the post-lanosterol cholesterogenesis, indicating that cAMP-dependent stimuli cross-talk with cholesterol feedback regulation. CRE-2 is essential for cAMP-dependent transactivation while SRE seems to be less important. Interestingly, when CREB is not limiting, the increasing amounts of SREBP-1a fail to transactivate the CYP51 promoter above the CREB-only level, suggesting that hormones might have an important role in regulating cholesterogenesis *in vivo.*

Introduction

Regulation of genes encoding enzymes of cholesterol biosynthesis and uptake is mediated generally by the negative cholesterol feedback loop and SREBP transcription factors. SREBPs need partners for performing their task of maintaining the cellular cholesterol level. One of the coregulatory signaling pathways is the cAMP-dependent pathway, which is characterized by the CREB/CREM/ATF1 family of transcription factors and protein kinase A (PKA). Induction of the cAMP pathway leads to phosphorylation of cAMP-dependent transcription factors, which bind to cAMP-responsive elements in the promoter/regulatory regions of the cAMP-responsive genes and in this way enhance their transcription (1). The induction of transcription can be immediate (few minutes/hours) or delayed (chronic - several hours). Genes that are rapidly and transiently induced in response to the intracellular signaling cascades are described as immediate early response genes (IEG). The induction of IEG occurs in the absence of *de novo* protein synthesis and thus could not be blocked by protein synthesis inhibitors. IEG encode secreted proteins, cytoplasmic enzymes and transcription factors (2). Most of our knowledge is about IEG encoding transcription factors c-jun and c-fos in the nervous and immune systems (3). The transcription of genes that respond to the induction by cAMP-dependent signaling is later repressed. The mediator of the repression is the cAMP-inducible transcription factor ICER, the inducible cAMP early repressor, synthesis of which is mediated by cAMP (4).

The SREBP regulatory pathway is characterized by sterol regulatory element binding proteins (reviewed in (5-8)). SREBP proteins are synthesized as membrane-bound inactive precursors whose processing is regulated by a lipid-sensor mechanism. Alteration in cholesterol content of membranes promotes changes in physical properties of the membrane in cholesterol (lipid)-rich conditions, which prevents the SCAP-SREBP complex from reaching the Golgi apparatus (9). INSIG was proposed to anchor the SCAP-SREBP complex in the ER in the presence of

cholesterol (10). Consequently, SREBPs remain membrane-bound precursors of the ER in lipid-rich conditions. The central dogma of cholesterol homeostasis says that when cholesterol, oxysterol and fatty acid levels are limiting (lipid-poor conditions), precursor SREBPs are transported to the Golgi apparatus and are proteoliticaly cleaved. The N-terminal soluble bHLH-Zip portions of mature SREBPs enter the nuclei and transactivate target genes by binding to sterol regulatory elements (SREs) of the target promoters. The exception is SREBP-2gc that is synthesized as an already soluble protein in male germ cells and can thus transactivate SREBP-dependent genes irrespective of the cell cholesterol level (11). Together with other data (12, 13), this has suggested that under certain physiological or pathophysiological conditions, the genes encoding cholesterogenic enzymes might more generally be up regulated irrespective of the cellular cholesterol level.

Lanosterol 14α-demethylase (CYP51) is an enzyme of the late portion of cholesterol biosynthesis. It represents the most evolutionarily conserved member of the cytochrome P450 (CYP) gene superfamily (14-16). The direct product of the CYP51 enzymatic reaction is follicular fluid meiosis activating sterol (FF-MAS), one of the short-lived intermediates of cholesterol biosynthesis that regularly accumulates only in testis and ovary (17).

Herein we demonstrate for the first time that forskolin, a mediator of the cAMP-dependent signaling pathway, activates the cholesterogenic *CYP51* with an immediate early time response also in the absence of mature SREBPs. A single CRE is sufficient to mediate this transactivation and ICER is involved in attenuation of transcription. The immediate early transactivation of the *CYP51* gene by forskolin is the first clear demonstration that cholesterogenic genes might respond to cAMP signaling also independently of the cholesterol feedback regulation. This might influence the *de novo* sterol synthesis when SREBPs are limiting. We used the model system of JEG-3 cells that are derived from an endocrine organ, the human placenta. These cells harbor the

components of cholesterol feedback regulation as well as of the cAMP-signaling pathway. Forskolin as a naturally occurring diterpene has been used to simulate the effects of physiologically occurring hormones (18) such are epinephrine, norepinephrine, FSH, LH, ACTH, glucagon, that are known to activate the cAMP-signaling pathway.

Materials and methods

Construction of Reporter Gene Plasmids - Preparation of the WT human CYP51 D7 CAT (-334/+316) has been described previously (12), as well as preparation of CYP51 D7 luc (-334/+316), CYP51 ΔD7 CAT (-121/+316), and CAT reporter mutant constructs CYP51-CRE2a- mut (TGACGCGA changed to TGATTCGA), CRE2b-mut (TGACGCGA was changed to AATGGCGA), CYP51-SRE1 mut (AATCACCTCAG was changed into TTTTTTTTTT) and CYP51-GC mut (GGGGGCGG was changed into GGGTTTTG) (13). Both CYP51-CRE-like mutants (CRE2a-mut and CRE2b-mut) have similar attributes in transfection studies (13). The substitution mutation CAT reporter constructs CYP51-CRE3-mut and CRE2-mut were prepared using QuickChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA). CYP51 D7 CAT served as a template for mutagenesis and preparation of CYP51-CRE3-mut, while for generation of a double mutant CRE2a-mut was used as a template.

Human *CYP51* C10 CAT (-471/+316) was amplified by the cloned *Pfu* Polymerase (Stratagene, La Jolla, CA) using sense 5'-TTTAG TTGGA GTGGG ACGGG TGACC-3' and antisense 5'-ACCTC GAACT GTGGC ACCTC ACCCT TCTCC-3' primers from the cosmid 121G12 containing the entire human *CYP51* gene (19). The fragment was cloned into the *SmaI*-digested pCATbasic plasmid (Promega, Madison, WI).

Mouse *Cyp51* CAT construct (-527/+104) was amplified by the cloned *Pfu* Polymerase (Stratagene, La Jolla, CA) using sense 5'-CCTTG AACCG ACGCG TGCCC AGAGG TGACG

TCTCC-3' and antisense 5'-CCTTG GCATC AAGAT CTCAT GGCCT GTCCG AGCAC C-3' primers from the BAC 51921 clone, containing the entire mouse *Cyp51* gene. This region of the Cyp51 promoter was cloned into the pCR-TOPO vector (Invitrogen, Carlsbad, CI) digested with *MluI* and *BglII*. The -527/+124 region of the mouse Cyp51 promoter was then cloned into the gel-purified p*CAT* basic from the *MluI* and *BglII* digested human *CYP51* D7 CAT. The -527/+124 region of the mouse Cyp51 promoter is analogous to the -334/+316 region of the human CYP51 promoter. All human and mouse *CYP51* CAT and luciferase reporter constructs were verified by sequencing.

Cell cultures - Human choriocarcinoma cells JEG-3 and human hepatocarcinoma cells HepG2 cells were cultured in DMEM media (Sigma, Taufkirchen, Germany) containing 5 % bovine calf serum and 1 % L-glutamine in a 5 % CO_2 incubator at 37^{o}C. In some experiments lipid rich media (COPUFA) has been used (DMEM with 1 % bovine serum albumin (BSA), cholesterol 10 µg/ml, 25-hydroxycholesterol 1 µg/ml, linoleic acid 0,15 mM) or 10 % delipidated serum (Sigma, Taufkirchen, Germany). In experiments with delipidated serum, the 10 % bovine calf serum medium was used as control (12). Cells were grown to 90 % confluency in T150 tissue culture flasks and were split 1:4 into 60cm^2 dishes 24 h prior transfections into normal or lipid rich media (COPUFA).

Transfections and determination of reporter gene activity - For transfection studies *CAT* and *firefly* luciferase reporter systems have been used. All transfections using *CAT* reporters were performed in human choriocarcinoma JEG-3 cells, while with human *CYP51* D7 luciferase reporter human hepatoma HepG2 and JEG-3 cells were transfected. Transfections and analysis of *CAT* (chloramfenicol acetyl transferase) activity have been performed as described previously

(12, 13). In experiments with deletion *CAT* constructs 3 μg of pSV CREMτ has been used. In experiments with mouse Cyp51 CAT construct, where interactions between SREBP and forskolin have been studied, 3 μg of pCMV SREBP-1a has been applied. In all other co-transfections studies 200 ng of pRSV CREB, pSV CREMτ, pCMV-SREBP1a and pSV-Sp1 have been applied. β-galactosidase has been used for normalization of the transfection efficiency with *CAT* reporters. pCAT basic was used as a DNA carrier to 20 μg.

Transfections of HepG2 cells or JEG-3 were performed with the *firefly* luciferase human *CYP51* D7 reporter construct. Cells were transfected with 2 μg of human *CYP51* D7 luciferase reporter, 500 ng of pSV β-galactosidase plasmid, and 200 ng of pSV CREMτ, pRSV CREB, or pSV ICERII, pSV Sp1 and pCMV SREBP-1a expression vectors or as it is indicated in the figure. pCAT basic was used as the carrier DNA to 3 μg. β-galactosidase was used for normalization of the transfection efficiency also in this case. The assay for determining the β-galactosidase activity was performed as described (20). *Firefly* luciferase activity was analyzed with the commercial kit for luciferase (Promega, Madison, WI) and Turner TD-20/20 luminometer. All transfection experiments were performed at least three times with two petri dishes for each experimental condition. In all transfection experiments, reporter activity has been calculated by the formula: *reporter activity = (normalized reporter gene activity)/(reporter activity in non-treated cells).* Reporter activity in non-treated cells represents unit 1 and is shown as a black bar in column 1 of each diagram. The average value, SEM and p values from two-tailed t test were calculated with the Excel program (Microsoft Corp., Redmond, WA). pSV CREMτ and pSV ICERII were a gift of dr. P. Sassone-Corsi (CNRS, Strasbourg, France). pRSV CREB and pSV Sp1 originate from the laboratory of dr. M. R. Waterman (Vanderbilt University, Nashville, TN, USA). It was

confirmed previously that the results of transfections of human *CYP51 CAT* and *firefly* luciferase reporter genes are comparable (Ačimovič et. al., unpublished).

Nuclear extract preparation, expression and purification of recombinant CREMτ protein and gel shift analysis - Nuclear extracts were isolated from JEG-3 cells that were grown in normal and COPUFA media as described above. Medium was changed every 24 h. Nuclear extracts were isolated from four petri dishes after 72 h, as described previously (12).

Purification of CREMτ expressed in bacteria has been described previously (21). Oligonucleotides used to generate double-stranded fragments containing human, mouse and rat *CYP51*-CRE and human *CYP51*-SRE1 element are presented in Table 1. Gel shift experiments were performed as described previously (12, 13).

RNA isolation and quantification of CYP51 mRNA by real time PCR and northern analysis - Total cellular RNA was an isolated form JEG-3 cell. 48 h before the beginning of the experiment, cells were split 1:5 and were subjected to normal or lipid rich media COPUFA. Media were replaced every 24 h. After 15 min, 0,5 h, 1,5 h, 2 h, 4 h, 8 h, 12 h, 16 h, 24 h, 36 h, 48 h, 50 h, 52 h, 56 h and 60 h and the media was aspirated. Cells were washed twice with RNAse-free PBS buffer, harvested with a rubber policeman and total RNA isolated with TRI reagent (Sigma, Taufkirchen, Germany). At 48 h the medium was changed into fresh COPUFA medium and cycloheximide (CHX) was added (10 μg/ml final concentration). 15 min after the addition of CHX forskolin (25 μM final concentration) has been added. Cells were harvested after 15 min, 30 min, 1 h, 1,5 h, 2 h, 4 h, 8 h and 12 h, and RNA was isolated as described above**.**

Total RNA from two 55 mm petri dishes was combined in each experiment. RNA concentration and quality were determined by RNA 6000 Nano Assay with Agilent 2100 Bioanalyzer (Agilent Technologies). At least two different samples of each RNA sample have been isolated and investigated for each time point.

1 μg of total RNA was converted into cDNA in a 20 μl reaction mixture using a SuperScript II Reverse transcriptase (Invitrogen, Carlsbad, CI) with random primers (Promega, Madison, WI). Reaction mixture was treated with DNase I (Sigma, Taufkirchen, Germany) to remove the contaminating DNA. The quality of each cDNA was tested in PCR reaction with β-actin primers.

Real time PCR was performed with the ABI PRISM 7900 HT Sequence detection system (Applied Biosystems, Foster City, CA) applying TaqMan technologies with the Assay by design human CYP51 primers sense 5'-CAG GTT GGC TGC CTT TGC-3' and antisense 5'-CTT GAT TTC CCG ATG AGC TCT GT-3', and probe CCC TGC GTC TGA AAC T labeled with FAM.

The expression of the *CYP51* has been normalized to 18S rRNA content using a TaqMan Ribosomal RNA Control Reagent Kit. Each 20 µl reaction mixture contained 1 µl of 100 x diluted cDNA template, 10 µl TaqMan Universal PCR Master Mix, 8 µl of water and 1 µl of 20 x control 18S rRNA or investigated CYP51 primers and probe, and was amplified as follows: after incubation at 50ºC for 2 min and denaturating at 95ºC for 10 min, 50 cycles were performed at 95ºC for 15 s, and 60ºC for 1 min. The level of each cDNA was determined by the comparative C_T method as described in User Bulletin 2, 1997. All procedures followed the Applied Biosystems (Foster City, CA) protocols. For each time point real time PCR was conducted in triplicates from at least two independently isolated RNA sample. All calculations (average C_T values, $\Delta\Delta C_T$, average values, SEM and p values from two-tailed t test) were calculated with the Excel program (Microsoft Corp., Redmond, WA).

Northern blot analysis was performed by standard procedures (20). ^{32}P-labeled 449 bp probe from the coding region of the human *CYP51* gene (exons 1 - 3) was prepared with Prime-It II random primer labeling kit (Stratagene, La Jolla, CA). Filters were hybridized for 2 h with QuikHyb hybridization solution (Stratagene, La Jolla, CA), washed twice for 15 min at room temperature (0,1% SDS, 0,3 M NaCl, 0,03 M sodium citrate), once for 30 min at 60°C (0,1% SDS, 0,3 M NaCl, 0,03 M sodium citrate) and exposed for 16 h to 48 h.

Immunoblot analysis - Western blot analysis was performed with 20 µg of nuclear proteins isolated from JEG-3 cells after forskolin treatment in different time points and analyzed using an anti-CREM polyclonal antibody (21) and visualized by the SuperSignal West Pico Chemiluminescent Substrate (Pierce, Rockford, Il, USA).

Metabolic labeling and sterol analysis - JEG-3 cells were split 1:2 the day before the beginning of the experiment. Cells were split 1: 6 into 75 cm^2 cell culture flasks at day 0 and 10 ml of normal media was added. On day 1, media has been changed to normal media or in lipid rich media (COPUFA media). After 48 h (day 3) ketoconazole (1 μM final concentration) and/or forskolin were added (25 μM final concentration) together with the mixture of 80 μCi [^{3}H] sodium acetate (NEN life Science products, Boston, MA, USA, 10 mCi/ml, 3,1 Ci/ mmol) and 0,2 μmol of cold sodium acetate per 1 ml of media.

Cells were returned to cell culture incubator for additional 8 h. After trypsinization and homogenization, the concentration of total cellular proteins was determined in the homogenate and sterols extracted from 0,8 ml of the homogenate in glass vials. 10 μl of ergosterol in ethanol (0,5 mg/ml), 100 μl of 0,3 M NaH_2PO_4 (pH = 1,0) and 3 ml of extraction solution (75% n-heptane, 25% isopropanol) have been added. The vials were incubated for at least 2 h on a shaker in dark at room temperature and centrifuged (2000 g, 10 min, RT). Organic phase was transferred and dried under vacuum, washed with 2 ml of n-heptane and centrifuged. Supernatants were transferred into fresh tubes and dried again. Samples were dissolved in the weight quantity of HPLC solvent. HPLC analysis was performed as described previously (22). Cholesterol fraction contains up to 20 % of lathosterol. Post-lanosterol intermediates 7-dehydrocholesterol, zymosterol and desmosterol eluted at identical retention time under applied separation conditions. Sterols standards (cholesterol, lanosterol, 7-dehydrocholesterol, zymosterol, desmosterol) were from Steraloids (Newport, Rhode Island).

The average values, SEM and p values from two-tailed t test were calculated with the Excel program (Microsoft Corp., Redmond, WA).

Results

CYP51 mRNA expression pattern in lipid-repressed and cAMP-induced conditions follows the immediate early response

The cAMP-signaling pathway is one of the most investigated and in biological systems one of the most frequently used pathways mediating cellular responses to different physiological stimuli, but its involvement in cholesterol homeostasis has been suggested only recently. The aim of our work was to investigate the molecular mechanisms of cAMP-mediated transcription of cholesterogenic *CYP51* that participates in late phases of cholesterol biosynthesis. *CYP51* is transactivated by transcription factors of the SREBP family (12, 13) in a similar manner as other cholesterogenic genes. In accordance with the cholesterol feedback regulation, the amount of nuclear SREBPs is highest when cholesterol, oxysterols and polyunsaturated fatty acids (COPUFA) are at minimum (7, 23), and is decreased in lipid-rich media (23). Fig. 1A shows that lipid rich medium COPUFA, containing 25-hydroxy-cholesterol, cholesterol and linoleic acid, prevents the formation of a specific SREBP-dependent transactivation complex at the CYP51 promoter. A 22 bp CYP51-SRE1 element of the human CYP51 promoter binds SREBPs specifically (Fig. 1A). Addition of SREBP-1a antibodies prevented binding of nuclear proteins to the human CYP51-SRE1 (compare lanes 1,2). No binding to the CYP51-SRE1 was detected in nuclear extracts from the COPUFA medium (lane 3). Results indicate that lipid rich medium abolishes SREBP binding to CYP51-SRE1. Fig. 1B shows a gradual decrease in CYP51 mRNA in lipid rich medium (0 – 12h), which was studied in detail by real time PCR (Fig. 1C). The level of CYP51 mRNA is reduced by 60 % in 2 h and 90 % in 12 - 16 h, and is later stabilized at about 30 % for the entire time of the experiment. Data of Fig. 1 thus show that no SREBPs are bound to the CYP51-SRE element in COPUFA medium and that this results in a marked decrease of CYP51 mRNA. If under such conditions cells are treated with forskolin, a mediator of cAMP response, the CYP51

mRNA level raises again, in a transient fashion. 48h of COPUFA treatment represents also time 0 of forskolin addition. Forskolin, in the presence of COPUFA, increased the CYP51 mRNA in 1.5 - 2 h (49.5 – 50h in Fig. 2A). After this time the CYP51 mRNA progressively decreased to the basal level in 4 - 6 h (52 – 54h in Fig. 2A). The addition of CHX, an inhibitor of protein synthesis, not only abolishes the decrease in CYP51 mRNA accumulation with time but allows the increase to continue (Fig. 2A). These results show that C*YP51* transcription is induced by cAMP-dependent agents in an immediate-early fashion, and that for such activation *de novo* protein synthesis is not needed. However, *de novo* protein synthesis is required for attenuation of transcription.

To monitor, whether the forskolin-induced *CYP51* transcription affects also sterol synthesis, JEG-3 cells have been metabolically labeled with 3H-acetate, a C-2 precursor of cholesterol. Addition of forskolin to JEG-3 cells leads to a statistically significant decrease in the CYP51 enzyme substrate lanosterol after addition of forskolin, while the quantity of post-lanosterol intermediates and cholesterol did not change significantly (Fig. 2B), which can be due to the well-known forskolin-induced consumption of cholesterol in the synthesis of steroid hormones (24, 25). Data indicate that the forskolin-mediated activation of *CYP51* transcription leads to an increased activity of the CYP51 protein followed by an increased expenditure of lanosterol. Addition of COPUFA medium reduced the cholesterol quantity 10-fold, and the lanosterol quantity for almost 6-fold, reaching the detection limit of the method (data not shown).

To show, that the decrease in lanosterol quantity is a result of an increased consumption of lanosterol and not a decreased production of lanosterol due to a forskolin-mediated inhibition of an enzyme of the pre-lanosterol cholesterol biosynthesis, a CYP51 inhibitor ketoconazole has been applied (Fig. 2B). Treatment with ketoconazole increased significantly the quantity of lanosterol (black bars) and 24,25 dehydrolanosterol (not shown) while the post-lanosterol

intermediates have been below the level of detection both in normal and in the COPUFA medium. Appearance of 24,25 dehydrolanosterol, another possible substrate of CYP51 in cholesterol biosynthesis, indicates that ketoconazole completely blocked CYP51 and that cholesterol biosynthesis did not take place by an alternative pathway. This is in accordance with the belief that a block in any of the cholesterol biosynthesis steps results in the absence of all downstream pathway intermediates. ICER is a cAMP-inducible repressor that is encoded by the last five exons of the *CREM* gene and is transcribed form the intronic *CREM* promoter P2 upon cAMP induction (4). ICER lacks the transactivation domain but contains the DNA binding domain, thus representing a cAMP-dependent repressor that binds to identical cAMP-responsive elements as the activatory forms of CREB and CREM. The addition of forskolin results in a gradual increase of the cAMP-inducible repressor CREM isoforms ICER II and ICER IIγ while significantly higher molecular weight activatory CREM isoforms (CREM τ, α, γ and S) do not fluctuate majorily during the treatment (Fig. 3A), which is consistent with the lack of inducibility of non-ICER CREM transcripts (4). Overexpression of pSV ICER repressed the human *CYP51* D7 luciferase reporter gene activity while overexpression of pRSV CREB induced the *CYP51* D7 luciferase reporter (Fig. 3). ICER represses the CREB-induced transactivation of CYP51, as lower luciferase activity was determined when ICER and CREB have been coexpressed in comparison to the overexpression of CREB alone ($p < 0,05$). This is consistent with the CYP51 mRNA pattern of Fig. 2A.

Evaluation of CRE elements in promoters of mammalian CYP51 genes

Mammalian CYP51 promoters are evolutionarily conserved and contain three cAMP-responsive elements CRE1, CRE2 and CRE3, which all differ from the consensus CRE. Only the human and

mouse CYP51-CRE1 are capable of binding the recombinant CREMτ (Fig. 4A, columns 1 and 2) while the rat *CYP51*-CRE1 failed in binding this cAMP-dependent transcription factor (Fig. 4A, column 3). The sequences of individual CRE-like elements (Fig. 4B) show that the downstream portion of the CYP51-CRE1 palindrome is not conserved. In contrast to CRE1, CYP51-CRE2 and CRE3 elements are conserved and are identical in human/pig and in mouse/rat CYP51 promoters (Fig. 4B).

To explore further the importance of individual CREs, the human CYP51 promoter deletion CAT constructs have been transfected into JEG-3 cells together with overexpressed CREMτ. Results demonstrate that the shortest construct ΔD7 exhibits a very low basal activity (5 % of D7) and does not respond to overexpression of CREMτ (Fig. 5B, column 1). This indicates that the -121/ +316 human CYP51 promoter is not sufficient for basal transcription and is unable to respond to cAMP-dependent stimuli despite the presence of CRE3.

Interestingly, the longest *CYP51* promoter construct C10 (Fig. 5B, column 3) also exhibits a lower basal activity (25 % of D7) and shows a weaker response to CREMτ despite the presence of three CRE-like elements in comparison to D7, containing CRE2 and CRE3 only (Fig. 5B, column 2).

The -471/-334 region including CYP51-CRE1 seems not to be important for the cAMP-dependent transactivation of *hCYP51*, or the repressor element might be present in this region, since no additional activation has been observed in the *CYP51* C10 construct that contains CRE1 in addition to CRE2 and CRE3.

To evaluate further the hypotheses based on deletion promoter-reporter construct, promoters with mutant CRE2 and CRE3 regulatory elements have been transfected together with over expressed CREMτ or CREB. While the mutation of CRE2 abolished both basal expression and the cAMP-

dependent transactivation, CRE3 mutation retained approximately half of CREMτ-dependent and over 60 % of CREB-dependent transactivation (Fig. 5C). As expected, the double mutation exhibits a similar response as the CRE2-mutant alone. The cumulative results of Figs. 5B and C suggest that the human *CYP51*-CRE2 is the principal CRE-like element responsible for cAMP-dependent transactivation. Interestingly, CRE3 has a strong effect on the basal activity of the CYP51 reporter, suggesting potential interactions with the basal transcriptional machinery.

cAMP-dependent transactivation of human and mouse CYP51 and cross-talk with the sterol regulatory pathway

The -334/+316 human and -527/+104 mouse CYP51 promoters, both containing CRE2 and CRE3 elements, have been applied in functional promoter studies in JEG-3 and HepG2 cells with overexpressed CREMτ and CREB. CAT activity of the human and mouse CYP51 promoters in normal medium was taken as "basal" and all modifications, such as change of the medium or overexpression of transcription factors, were compared to the CYP51 promoter - *CAT* reporter activities observed in the normal medium.

Generally, both human and mouse *CYP51*-CAT constructs show a similar pattern of response (transactivation) to overexpression of CREMτ and CREB in individual cell lines. In normal medium, the overexpression of CREMτ and CREB results in up to 3-fold activation of *hCYP51* in HepG2 cells and 4 - 12-fold activation in JEG-3 cells (Fig. 6A). The mouse Cyp51 promoter is also transactivated by overexpressed CREMτ and CREB, albeit to a lower extent compared to the human CYP51 promoter. The observed weaker response of the mouse Cyp51 promoter in a human cell line nuclear environment might be explained by the requirement for an optimal species-specific transactivation complex. Several gene-specific transcription factors as well as

factors associated with the general transcriptional machinery differ between human and mouse in amino acid sequence and consequently also in the three-dimensional structure. For example, transcription factor SREBP-1a is 80.4 % identical between mouse (NP_035610) and human (NP_004167) while CREMτ seems to be more conserved and shows a 94.2 %, identity between mouse (P27699) and human (Q03060). Numbers in brackets represent Genbank identification numbers.

The transactivation capacity of CREMτ and CREB was also monitored in lipid-rich medium COPUFA (Fig. 6B). As shown previously (Fig. 1A), COPUFA prevents binding of SREBP to CYP51-SRE. Fig. 6B shows that treatment of cells with COPUFA results in a decrease of human (7-fold in HepG2 and 6-fold in JEG-3 cells) and mouse (20 fold in JEG-3 cells) *CYP51*-CAT reporter activities. Overexpression of CREMτ and CREB results in re-activation of CYP51 promoters of both species in lipid rich medium, 1.5-fold (CREMτ) or 3.5-fold (CREB) above the basal level for the human promoter.

The effect of forskolin on the SREBP-1a mediated transactivation is shown in Fig. 6C. Lanes 1 – 3 show the effect of decreasing amounts of lipids (COPUFA, normal medium, delipidated serum) on the CYP51-CAT reporter transactivation. Overexpression of nuclear SREBP-1a (lanes, 4,5) amplifies transactivation. No major difference is observed between cells grown in normal serum and delipidated serum. When forskolin is added to the cells with overexpressed SREBP-1a, the transactivation is diminished (compare last two lanes). This suggests interactions and/or competition for binding sites of CRE-2 and SRE-1 factors, since the core elements (CRE-2 octamere and SRE-1 decamere) are separated for only 12 bp (13). The titration assay, where different concentrations of SREBP-1a and CREB expression plasmids were co-transfected to JEG-3 cells together with the human CYP51-luciferase reporter (Fig. 6D) was used to evaluate

potential interactions between CRE-2 and SRE-1 binding factors. At low CREB concentrations (0 - 20 ng of CREB expression plasmid), increasing amounts of SREBP-1a increase the transactivation of the CYP51 promoter. The transactivation is highest when 600 ng of SREBP-1a plasmid and no CREB are expressed. Similarly, at low SREBP-1a concentrations (0 - 20 ng of SREBP-1a expression plasmid), increasing amounts of CREB increase the transactivation of the CYP51 promoter. The maximal CREB-dependent transactivation of human *CYP51* is lower compared to the SREBP-dependent transactivation. Interestingly, increasing amounts of CREB decrease the maximal SREBP-dependent transactivation, suggesting that CRE-dependent transcription factors bind with a higher affinity to the CYP51-CRE2 site. When CREB is not limiting (at least 200 ng of the expression plasmid), even increasing amounts of SREBP-1a cannot transactivate the promoter above the CREB-only maximal level. This suggests that at high concentrations the CYP51-CRE2-bound CREB might be able to displace SREBP-1a from the CYP51-SRE1. Alternatively, CREB protein interaction with SREBP-1a might lead to a less potent transactivation complex, resulting in less efficient transciption of the CYP51 gene. Similar results were obtained for CREMτ, however 600 ng of the pRSV CREMτ expression plasmid did not yet saturate the promoter (not shown). It seems possible that at physiological conditions that result in high concentrations of cAMP-dependent transcription factors, the cholesterogenic *CYP51* responds as a cAMP-dependent gene irrespective of the SREBP signaling pathway. Further studies are required to evaluate this hypothesis and to depict the endogenous concentrations of interacting CRE-binding factors and SREBPs.

Relative importance of the CYP51 promoter elements for the cAMP-mediated transactivation

Data above show CRE2 as most important for the cAMP-dependent transactivation of CYP51. This element lies within the -182/-125 promoter region that contains also GC-box and SRE-1

elements (13). In the normal medium all three mutants (CRE2, SRE and GC-box) exhibit a lower basal activity compared to the wild type human *CYP51* CAT reporter (Figs. 7A, B). The highest effect of the mutation is observed with mutated GC-box where only 11 % of the basal activity was retained, while CRE2b and SRE1 mutations retained about 45 % of the basal activity. CRE2b mut showed the weakest response with only 12 % of the wild type reporter activity after overexpression of CREB. 50 % of CAT activity remains after overexpression of CREMτ with the SRE-1 mutant and 37 % after overexpression of CREB. GC-box mut preserved only 10 % of the CREMτ-dependent transactivation and 30 % of the CREB-dependent transactivation.

The data in Figs. 7A, B demonstrate that the three DNA regulatory elements (GC-box, CRE2 and SRE1) from the –282/-209 region of the human CYP51 promoter contribute to the basal as well as cAMP-dependent *CYP51* gene transactivation in normal medium that contains SREBPs from the bovine serum. While an intact GC-box seems to be most important for the basal transcription, CRE2 is essential for the CREMτ/CREB-dependent transcription, while SRE1 seems to have a lower impact. As established previously, SRE1 has a major role in SREBP-dependent transactivation of CYP51 (13).

COPUFA diminished the activity of the wild type human *CYP51* CAT reporter to 16 % (Fig. 7C). The activities of CRE2 mut and GC-box mut were diminished to 50 % of the initial value in normal medium. The CYP51 SRE1 mut reporter activity is diminished to 45% of the wild type in the presence of SREBPs from the normal, bovine serum-supplied, medium. Additionally, our previous results have shown clearly that in the presence of overexpressed SREBP-1a, the CYP51 SRE1 mut activity is diminished t o10% of the wild type (13). The human *CYP51* SRE1 mut has a similar response as the wild type promoter in sterol-rich media, particularly with CREM-

depenendent transactivation, suggesting activation by CREM in the absence of SREBP-dependent transactivation complex.

Overexpression of Sp1 does not affect majority the *CYP51* transactivation in normal media nor in the lipid rich conditions (Figs. 7 B, C gray bars). This observation is in accordance with the previously published work (13), however, it seems to be controversial with dramatic effects observed with mutations of the GC-box. This seeming discrepancy can be explained by the ubiquitous nature of Sp1 that is likely not limiting in JEG-3 cells. Alternatively, Sp1 might serve as a competence factor. For example, overexpression of a competence factor may result in no basal induction of promoter activity but its binding to response elements may still be required to potentiate transactivation by other factors.

Discussion

Being a part of the cholesterol biosynthesis pathway, *CYP51* transcription is regulated primarily by transcription factors SREBP. Cholesterol/SREBP dependent regulation of *CYP51* has been demonstrated previously by several examples (8, 12, 13, 28-31). Unsaturated fatty acids and 25-hydroxycholesterol work synergistically in lowering the nuclear, transcriptionally active forms of SREBP-1 and SREBP-2 proteins (32) by inhibiting the SREBP precursor cleavage and its translocation to the nucleus. Repressing effect on SREBP-regulated genes has been detected already by 16 h and has been preserved for 48 h at the level of transcription (33-37). The *CYP51* transcription is reduced by over 50 % within 4 h in the lipid-rich COPUFA medium. This quick repression could be explained with the short half-life of the nuclear forms of SREBP. Wang and coworkers reported that reduction at the level of nuclear forms of SREBPs is observed already 30 min after addition of cholesterol and 25-hydroxycholesterol (38). Degradation of SREBPs requires protein synthesis and can be blocked by the inhibitor of protein synthesis CHX. Since in

the presence of CHX the degradation of SREBPs is blocked, the transcription of SREBP target genes is slightly up-regulated (39). Similar observation has been noticed also in our research (Fig. 2A, 52 h).

In this work we describe for the first time an immediate early transactivation of the cholesterogenic *CYP51* by a cAMP-mediator forskolin. Reports from the late eighties have indicated that the LDL receptor (36, 40) and HMG-CoA reductase (36, 37) mRNA levels are elevated by cAMP-dependent agents, however, the mechanisms have not been investigated. The immediate early cAMP-dependent response described in this work does not depend on the cellular lipid level, showing that at least some cholesterogenic genes can become transactivated by other stimuli, also irrespectively of the cholesterol feedback regulation. When phosphorylated CREB binds to cAMP-responsive elements, it regulates immediate early genes, such as *c-fos* and *junB* (41). Together with other immediate early genes, the expression of the inducible cAMP early repressor ICER (42, 43) mRNA is upregulated after 1.5 h of forskolin treatment, reaching maximal levels after 3.5 h. This rise in expression of ICER correlates well with the decrease in *c-fos* and *junB* levels (44), as well as with time and concentration dependency of the repression of CRE-mediated *luciferase* gene expression after the treatment of CHO cells with forskolin (45). Our results show that the *CYP51* gene responds to activation by the cAMP-signaling pathway in the same immediate-early time-dependent manner. Highest level of CYP51 mRNA is detected 1.5-2 h after addition of forskolin to JEG-3 cells grown in lipid rich medium COPUFA (Fig. 2A) as well to cells grown in normal medium (data not shown) and returns to basal level after 4 h. Early response does not require protein synthesis in contrast to the delayed cAMP response (46, 47). In our case, the addition of a protein synthesis inhibitor CHX did not prevent the induction of *CYP51* transcription, suggesting that *CYP51* transactivation does not depend on *de novo* protein synthesis. However, the attenuation of *CYP51* transcription likely requires *de novo* synthesis of

the cAMP-inducible repressor ICER. It seems that the declination of *CYP51* transcription is due to the synthesis of ICER which binds to CRE elements in the promoters of cAMP-responsive genes and represses their transcription (4, 48). ICER is transcribed from a cAMP-dependent intronic promoter of the *CREM* gene. It binds identical CRE elements as the activator CREM and CREB isoforms. However, ICER does not contain a transactivation domain. It displaces the activator transcription factors from CRE elements in the cAMP-dependent promoters and thus acts as a transcriptional repressor (4). As shown in Fig. 3A, ICER synthesis was induced in a time-dependent manner also in our experiments, in accordance with the attenuated CYP51 mRNA level (Fig. 2A). Overexpression of ICER presumably repressed the CREB mediated transcription of *CYP51 D7* luciferase reporter construct (Fig. 3B), suggesting competitive displacement of CREB by ICER. Even if mammalian CYP51 promoters contain three potential cAMP-response elements, it seems that only CYP51-CRE2 is responsible for the immediate early response of *CYP51*, including the cAMP-mediated transactivation as well as repression. This conclusion arises from the following observations: 1 - CRE2 is evolutionarily the most conserved CRE-like element in the four mammalian species; 2 - mutation of CRE2 abolished the cAMP-dependent response of the human *CYP51* gene; 3 - the longest promoter - reporter construct, containing CRE1, CRE2 and CRE3 has a weaker cAMP-dependent response compared to the construct containing CRE2 and CRE3 only; 4 - the shortest promoter-reporter construct containing only CRE3 does not respond to cAMP-dependent transactivation. Interestingly, the shortest -121/+316 ΔD7 construct that contains the downstream promoter element (DPE) and the initiator element (INR) of the four studied mammals (49), shows below 10 % of the basal activity compared to the -334/+316 promoter. This suggests that in the case of mammalian TATA-box lacking CYP51 promoters; the DPE and INR seem not to serve as the RNA pol II complex binding sites.

These results suggest that *CYP51* belongs to the group of immediate early response genes. Importantly, forskolin by modulating the transcription of *CYP51* modulates also the pre-cholesterol sterol synthesis in JEG-3 cells *ex vivo*. While the quantity of the *de novo* synthesized lanosterol seems to decrease, the quantity of post-lanosterol intermediates of cholesterol biosynthesis did not change or did even increase in conditions when cells have been treated with forskolin. This suggests an increased consumption of lanosterol to form post-lanosterol intermediates. Reasons for a statistically non-significant increase in post-lanosterol intermediates might be due either to the low activity of enzymes of the post-lanosterol phases of cholesterol biosynthesis or to an increased expenditure of the synthesized cholesterol. It is well known that forskolin increases the expenditure of cholesterol since genes that encode enzymes involved in production of steroid hormones are highly up regulated by the cAMP-signaling pathway (24, 25). Identification of all possible steroid metabolites that are derived from cholesterol after the forskolin-mediated immediate early activation in JEG-3 cells is beyond the scope of this work.

Our data show for the first time that a cholesterogenic gene *CYP51* can be activated transcriptional through CYP51-CRE2 in an immediate early fashion after forskolin treatment, avoiding the cholesterol feedback repression. Either CREB or CREMτ can mediate this transactivation and ICER is responsible for attenuation of transcription. The forskolin stimulus leads to a short-term transactivation of cholesterogenic *CYP51* that is reflected also at the level of *de novo* synthesis of cholesterogenic sterol intermediates.

In accordance with our data others have indicated that the cholesterol feedback regulation might be bypassed in some physiological or pathophysiological conditions, such as tumor development (50, 51), regeneration of tissues after injury (52-54), response to cytokines (52-56) or maturation of male germ cells (12). The high level of cholesterol in the cell presumably protects cells against stress while an increased cholesterol biosynthesis is one of the cellular responses to stress (57-

59). However, the biochemical details that would allow understanding these phenomena have been lacking so far. Our work shows that the *CYP51* gene is transcriptionally activated in sterol-repressed conditions by either CREB or CREMτ. While CRE2 and GC-box DNA elements are essential for cAMP-dependent transactivation, the SRE1 site seems to be less important. Transactivation of CYP51 through CRE2 represents the first clear evidence that the cholesterol feedback repression of cholesterogenic genes can be bypassed by transcriptional activation through another signaling pathway. Additionally, our work shows for the first time that a gene participating in cholesterol biosynthesis (*CYP51)* is regulated in an immediate early fashion, which influences the sterol profile of the cell. This opens new venues of research to better understand cholesterol-independent roles and cross talks of the cholesterol feedback regulation with other signaling pathways.

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Abbreviations

bHLH, basic-helix-loop-helix; BSA, bovine serum albumin; CAT, chloramphenicol acetyl transferase; CHX, cycloheximide; COPUFA, lipid rich medium containing cholesterol, 25-hydroxycholesterol and linoleic acid; CRE, cAMP response element; CREB, cAMP response element-binding protein; CREM, CRE modulator, CYP, cytochrome P450; CYP51, lanosterol 14α-demethylase; DPE, downstream promoter element; FF-MAS, follicular fluid meiosis activating sterol; gc, germ cell; HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A; HPLC, high performance/pressure liquid chromatography; ICER, inducible cAMP early repressor; IEG, immediate early response gene; INR, initiator element; LDL, low density lipoprotein; MAS, meiosis activating sterols; mut, mutant; PKA, protein kinase A; P450, cytochrome P450; PUFA, polyunsaturated fatty acid, Sp1; specificity protein 1; SCAP, sterol cleavage activating protein; SREBP, sterol regulatory element-binding protein; SRE, sterol regulatory element; 25-OH, 25-hydroxycholesterol; WT, wild type.

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Figure legends

Fig. 1. *The lipid rich medium COPUFA prevents the SREBP-dependent transcription of CYP51.* A. COPUFA prevents the binding of the SREBP-dependent transactivation complex to CYP51 SRE1. Gel shift experiment with CYP51 SRE1. Nuclear extracts were isolated from JEG-3 cells grown in normal medium (columns 1, 2) or COPUFA (column 3). Anti-SREBP-1 antibodies were added in column 1. Northern analysis (B) and real time PCR (C) of CYP51 mRNA after treatment with COPUFA. h – hours of treatment. mRNA was normalized to the quantity of 18S rRNA.

Figure 2. *Forskolin induces CYP51 transcription and increases expenditure of lanosterol.* A – Real time PCR of CYP51 mRNA in COPUFA medium after addition of forskolin (F) with or without cycloheximide (CHX). h – hours of treatment. mRNA was normalized to the quantity of 18S rRNA. B - Detection of 3H-labeled sterol intermediates with metabolic labeling after addition of forskolin (F) and/or ketoconazole (K). Lanosterol (black bars); post-lanosterol intermediates (a mixture of zymosterol, desmosterol and 7-dehydrocholesterol) - white bars, cholesterol (grey bar). Asterisk - $p < 0,05$. Follicular fluid meiosis activating sterol FF-MAS and testis meiosis activating sterol T-MAS were below the detection limit.

Figure 3. *ICER participates in forskolin-dependent CYP51 transcription.* A. Western blot experiment of nuclear extracts from JEG-3 cells with anti CREM antibodies. B. Transient transfections of human *CYP51 D7* luciferase reporter with overexpression of CREB (pRSV CREB) and ICER (pSV ICER).

Figure 4. A. *Recombinant CREMτ binds CYP51 CRE-like elements.* Mobility shift experiment with CREMτ and mammalian *CYP51* CRE-like elements: h - human, m - mouse, p - pig, r-rat. B. *The sequences of mammalian CYP51 CRE-like elements.* CRE sequences were included into oligonucleotides used in mobility shift experiments shown in Fig. 4A. Nucleotides that differ between mammalian CYP51 CREs are underlined.

Figure 5. *The dominant role of CYP51-CRE2-element in the cAMP-dependent transcription.* A. Scheme of the human CYP51 promoter/reporter constructs that were transfected into JEG-3 cells. DNA regulatory elements are framed. B. The *-334/-121 region is necessary for basal and cAMP-dependent expression of the human CYP51 gene*. JEG-3 cells were transiently transfected with *CYP51* deletion CAT constructs C10, D7, and ΔD7. Black bars - basal expression, white bars - overexpression of 3 μg of pSV CREMτ. C. *The role of CRE2 and CRE3 in cAMP-dependent transactivation.* Transient transfections with the human wild type *CYP51 D7* or mutated CRE elements. Black bars - basal expression, white bars - overexpression of 200 ng of pSV CREMτ., gray bars - overexpression of 200 ng pRSV CREB.

Figure 6. *Transactivation of the human and mouse CYP51 genes by CREB and CREMτ in normal(A) and COPUFA (B) medium.* The human *CYP51* D7 (-334/+316) and the mouse *Cyp51* D7 (-527/+104) reporters, transiently transfected into JEG-3 cells (human - white bars; mouse - gray bars) and HepG2 cells (human - black bars). *The effect of forskolin (C) and cross-talk with SREBP-1a (D)*. C - The mouse CYP51 promoter-reporter was transfected into JEG-3 cells grown in different media as indicated on the figure, with or without overexpressed SREBP-1a. D -The human CYP51 promoter-reporter was cotransfected with different amounts of pCMV SREBP-1a

and/or pRSV CREB expression plasmids (ng of plasmids). The average value of three transfections is presented on a three-dimensional diagram.

Figure 7: *The role of CYP51 promoter regulatory elements in the CREB and CREMτ-dependent transactivation in normal and COPUFA medium*. JEG-3 cells were transiently transfected with the human wild type CYP51 D7 CAT (A) or with CYP51 D7 CAT constructs with mutated elements CRE2, SRE1 and GC-box (B,C). Cells were grown in normal medium (A, B) or in COPUFA (C). CREM – overexpression of pSV CREM; CREB – overexpression of pRSV CREB, SREBP-1a - overexpression of pCMV SREBP1a. The COPUFA + Sp1 treatment represents the last bar of each group in Fig. 7C.

Table 1: *Pairs of oligonucleotides used to prepare probes for mobility shift studies and primers used for introduction of mutations into CAT reporters*. Bold – CYP51 CRE-like and SRE elements, underlined- nucleotides changed in the process of *in vitro* mutagenesis.

Probe	Sense primer 5'-3'	Antisense primer 5'-3'
CYP51 CRE1 human	GGGACGGGGC**TGACCTCA**CCGTCCT	AGGACGG**TGAGGTCA**GCCCCGT
CYP51 CRE2 human	GCCCCGC**TGACGCGA**TGTAGGCCGA	GATCTCGGCCTACA**TCGCGTCA**GCGG
CYP51 CRE3 human	GCCCCATTCTG**TGACGCAC**GGGGTGGC	CGCGCCACCCC**GTGCGTCA**CAGAATGG
CYP51 CRE1 mouse	GATTGTACGGG**TGATCCGA**TGGTCTC	GGGTGAGACCA**TCGGATCA**CCCGTAC
CYP51 CRE2 mouse	CCAGCTCTGC**TGACGCCA**CATAGGCC	CTCGGCCTATG**TGGCGTCA**GCAGAG
CYP51 CRE3 mouse	GCCCCGTCTG**TGACGTCC**TGGTGG	CGCGCCACCA**GGACGTCA**CAGACGG
CYP51 CRE1 rat	GGGCAATACCGC**TGAACCAA**CGGTCTCAC	GGGTGAGACCG**TTGGTTCA**GCGGTATTG
mut CRE2a human	GCCCAAGGCCCCGC**TGATTCGA**TGTAGGC	CGGCCTACA**TCGAATCAG**CGGGGCCTTG
mut CRE2b human	GCCCAAGGCCCCGC**AATGGCGA**TGTAGGC	GATCTCGGCCTACA**TCGCCATT**GCGG
mut CRE3 human	GCCCCATTCTG**TGATTCAC**GGGGTGGC	CGCGCCACCCC**GTGTTTCA**CAGAATGG
CYP51 SRE1	GGCCGAG**ATCACCTCAG**GCGCT	GCGAGCGC**CTGAGGTGAT**CTCGCG

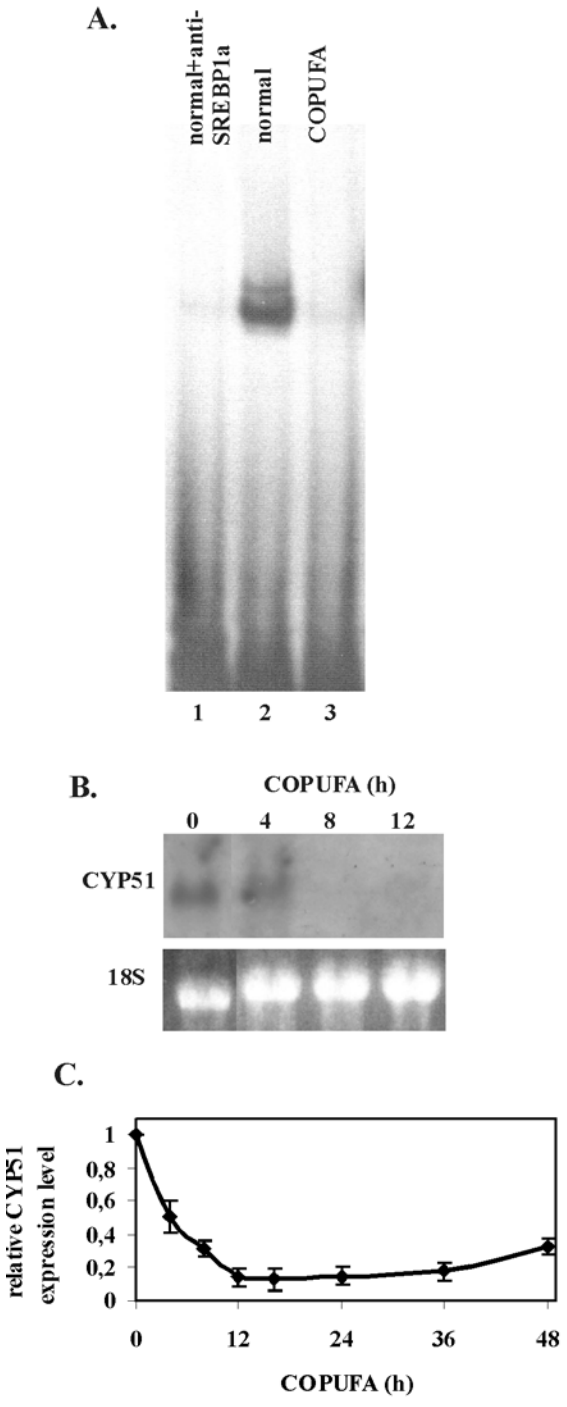

A.
normal+anti-SREBP1a
normal
COPUFA
1
2
3
B.
COPUFA (h)
0
4
8
12
CYP51
18S
C.
relative CYP51 expression level
1
0,8
0,6
0,4
0,2
0
0
12
24
36
48
COPUFA (h)

Fig. 1

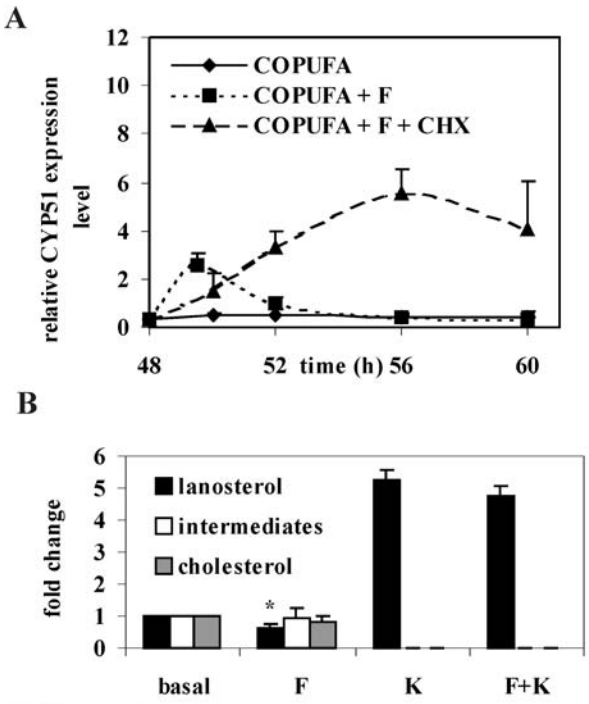

A
relative CYP51 expression level
12
10
8
6
4
2
0
COPUFA
COPUFA + F
COPUFA + F + CHX
48
52
time (h)
56
60
B
fold change
6
5
4
3
2
1
0
lanosterol
intermediates
cholesterol
*
basal
F
K
F+K

Fig. 2

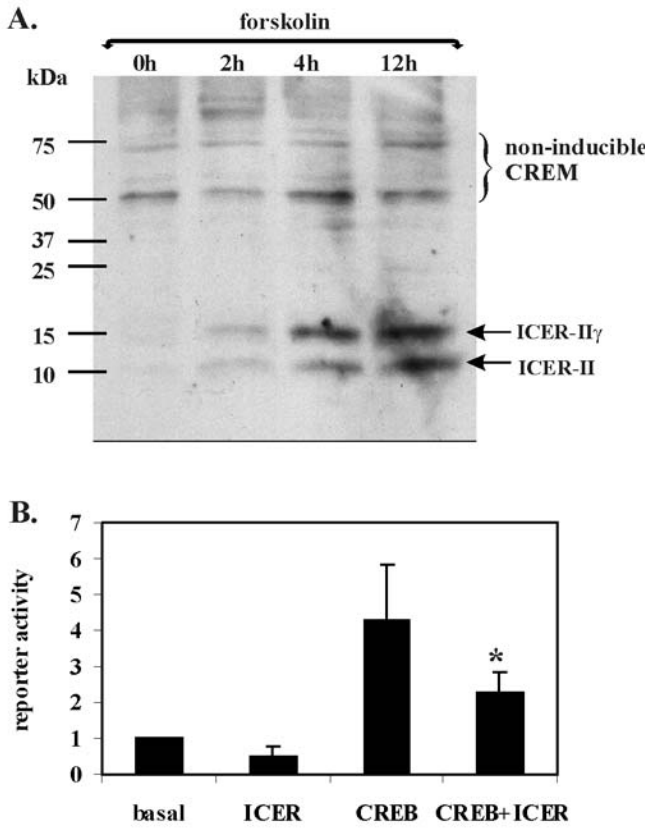
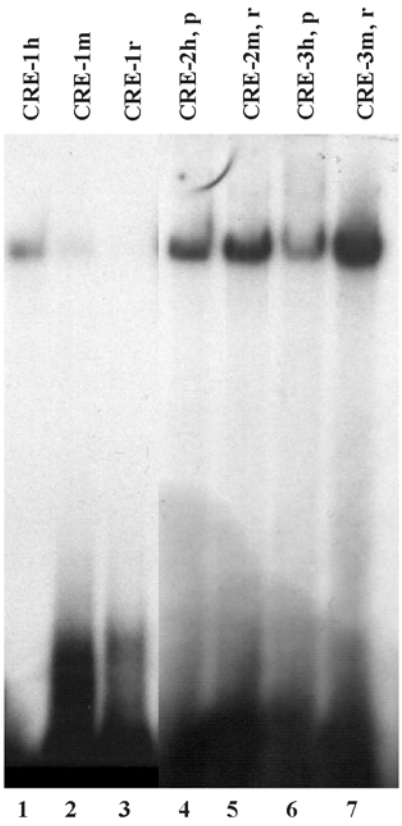

A.
forskolin
0h
2h
4h
12h
kDa
75
50
37
25
15
10
non-inducible
CREM
ICER-IIγ
ICER-II
B.
reporter activity
7
6
5
4
3
2
1
0
*
basal
ICER
CREB
CREB+ICER

Fig. 3

A.



B.

CRE1-h	TGACCTCA
CRE1-m	TGATCCGA
CRE1-r	TGACATTC
CRE2-h,p	TGACGCGA
CRE2-m,r	TGACGCCA
CRE3-h,p	TGACGCAC
CRE3-m,r	TGACGTCC

Fig. 4

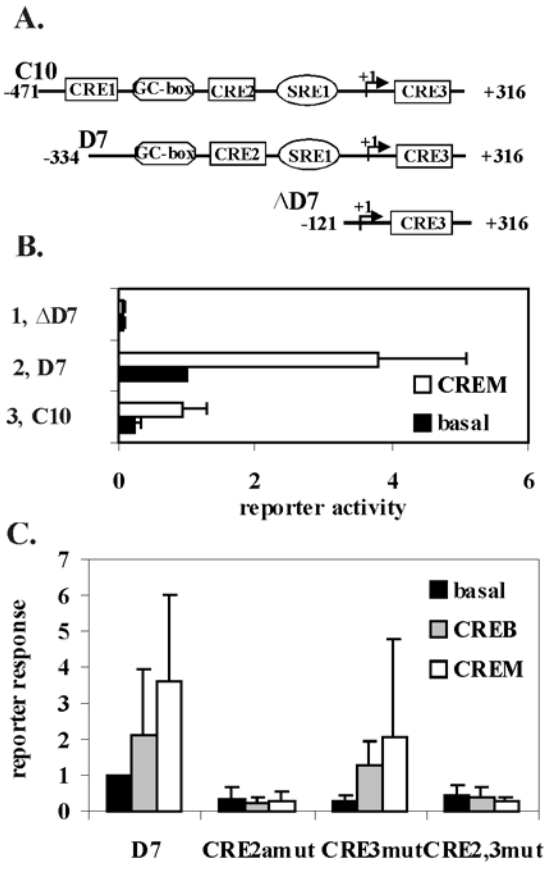

A.
C10
-471
CRE1
GC-box
CRE2
SRE1
+1
CRE3
+316
D7
-334
GC-box
CRE2
SRE1
+1
CRE3
+316
ΔD7
-121
+1
CRE3
+316
B.
1, ΔD7
2, D7
3, C10
CREM
basal
0
2
4
6
reporter activity
C.
reporter response
7
6
5
4
3
2
1
0
basal
CREB
CREM
D7
CRE2amut
CRE3mut
CRE2,3mut

Fig. 5

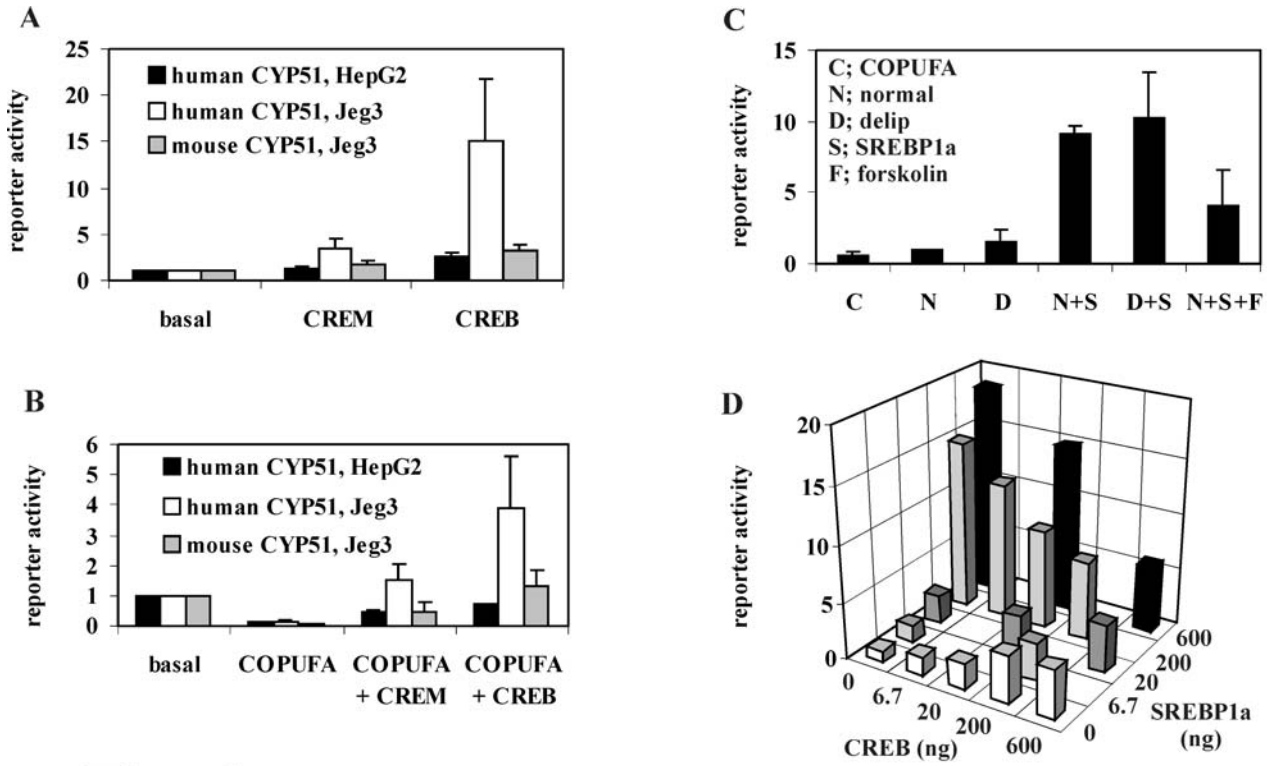

A
reporter activity
25
20
15
10
5
0
human CYP51, HepG2
human CYP51, Jeg3
mouse CYP51, Jeg3
basal
CREM
CREB
B
reporter activity
6
5
4
3
2
1
0
human CYP51, HepG2
human CYP51, Jeg3
mouse CYP51, Jeg3
basal
COPUFA
COPUFA + CREM
COPUFA + CREB
C
reporter activity
15
10
5
0
C; COPUFA
N; normal
D; delip
S; SREBP1a
F; forskolin
C
N
D
N+S
D+S
N+S+F
D
reporter activity
20
15
10
5
0
0
6.7
20
200
600
CREB (ng)
0
6.7
20
200
600
SREBP1a (ng)

Fig. 6

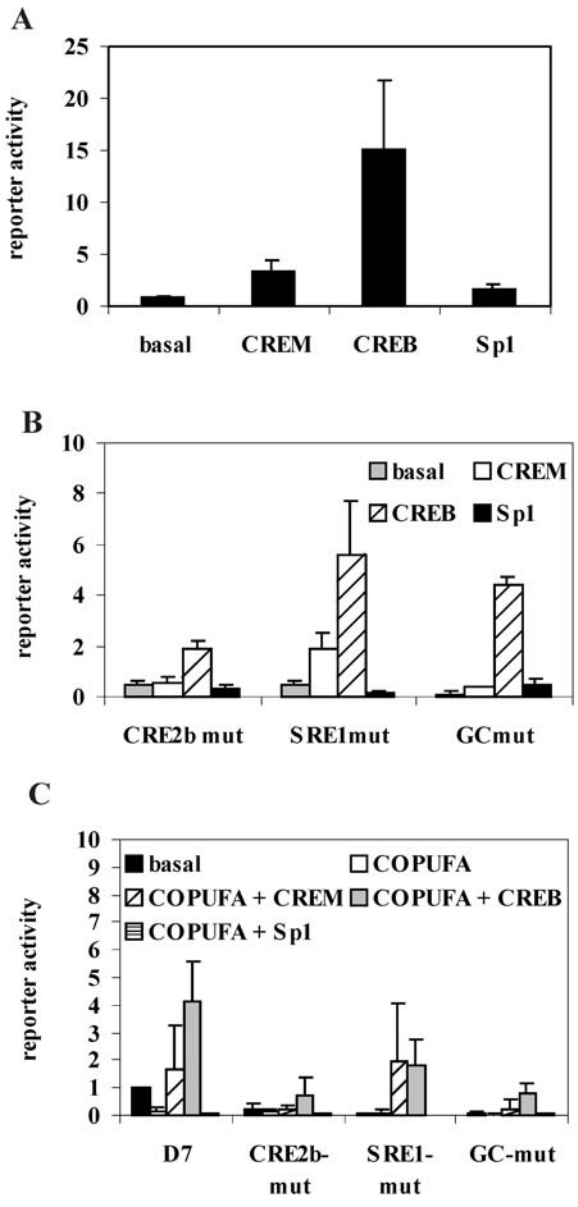

A
reporter activity
25
20
15
10
5
0
basal
CREM
CREB
Sp1
B
reporter activity
10
8
6
4
2
0
basal
CREM
CREB
Sp1
CRE2b mut
SRE1mut
GCmut
C
reporter activity
10
9
8
7
6
5
4
3
2
1
0
basal
COPUFA
COPUFA + CREM
COPUFA + CREB
COPUFA + Sp1
D7
CRE2b-mut
SRE1-mut
GC-mut

Fig. 7