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# Research Article

# Digoxin and ouabain increase the synthesis of cholesterol in human liver cells

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**Abstract.** Digoxin and ouabain are steroid drugs that inhibit the $Na^+/K^+$-ATPase, and are widely used in the treatment of heart diseases. They may also have additional effects, such as on metabolism of steroid hormones, although until now no evidence has been provided about the effects of these cardioactive glycosides on the synthesis of cholesterol. Here we report that digoxin and ouabain increased the synthesis of cholesterol in human liver HepG2 cells, enhancing the activity and the expression of the 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMGCR), the rate-limiting enzyme of the cholesterol synthesis. This effect was mediated by the binding of the sterol regulatory element binding protein-2 (SREBP-2) to the HMGCR promoter, and was lost in cells silenced for SREBP-2 or loaded with increasing amounts of cholesterol. Digoxin and ouabain competed with cholesterol for binding to the SREBP-cleavage-activating protein, and are critical regulators of cholesterol synthesis in human liver cells.

**Keywords.** Digoxin, ouabain, cholesterol, 3-hydroxy-3-methylglutaryl-coenzyme A reductase, sterol regulatory element binding proteins, SREBP-cleavage activating-protein.

## Introduction

Digoxin and ouabain are cardioactive glycosides with a steroid structure, and are widely used in the treatment of congestive heart failure and cardiac arrhythmias. By binding to specific extracellular sites of the $Na^+/K^+$-ATPase on the plasma membrane of cardiomyocytes, digoxin and ouabain inhibit the pump activity, thus increasing the intracellular $Na^+$ concentration and slowing down the extrusion of $Ca^{2+}$ by the $Na^+/Ca^{2+}$ exchanger. The higher intracellular $Ca^{2+}$ levels are thought to induce a positive inotropic effect on cardiac muscle [1].

Many lines of evidence suggest that $Na^+/K^+$-ATPase is not the only target of digoxin and ouabain. For instance, digoxin has been shown to regulate the transcription of several genes, such as cytochrome P450 CYP3A4, multidrug resistance-1 and steroid xenobiotic receptor, by unknown mechanisms [2]. Moreover, both digoxin and ouabain modulate the synthesis of corticosteroid hormones in human and rat adrenal glands [3–5], by regulating the activity of several enzymes, such as the steroidogenic acute regulatory protein and the cytochrome P450 side chain cleavage enzyme [3, 5]. These results are in keeping with other experimental observations, show-

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ing that the synthesis of steroid hormones is often controlled by the end products of the pathway or by endogenous/exogenous steroid compounds [6]. Owing to their steroid structure, cardioactive glycosides could be potential modulators of cholesterol synthesis, but presently no available data exist in this regard.

The rate-limiting step of the cholesterol synthesis is the reduction of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) to mevalonate, a reaction catalyzed by the HMG-CoA reductase (HMGCR; EC 1.1.1.34). HMGCR is inhibited by nanomolar concentrations of statins [7] and is strictly regulated with different mechanisms. The transcription of the HMGCR gene is under the control of sterol regulatory element binding proteins (SREBPs), which reside in the endoplasmic reticulum membrane in an inactive form. In sterol-depleted cells, a SREBP cleavage-activating protein (SCAP) binds the SREBPs and escorts them to the Golgi complex, where S1P and S2P proteases cleave them. After proteolytic cleavage, the SREBP N-terminal domain enters the nucleus, where it binds to the sterol-responsive element (SRE) of the HMGCR promoter, enhancing the gene transcription. In contrast, when intracellular cholesterol rises, the SREBP/SCAP complex is retained on the endoplasmic reticulum, SREBP is not cleaved and the transcription of genes involved in the cholesterol synthesis and uptake decreases [8, 9]. Amongst the three SREBPs isoforms (SREBP1a, 1c and 2), SREBP2 regulates preferentially the genes involved in the cholesterol homeostasis, whereas SREBP1a also controls the other SREBPs-regulated genes [6, 8].

At the post-transcriptional level, the activity and the amount of HMGCR enzyme may be reduced by a feedback inhibition exerted by metabolites derived from mevalonate [10] or by the ubiquitination and proteasomal degradation induced by cholesterol itself [11]. Phosphorylation on serine, which can be induced by hormones such as glucagon, is also known to decrease the activity of HMGCR [12].

In this work, we investigated whether the cardioactive glycosides digoxin and ouabain affect cholesterol biosynthesis in human cell lines and, if so, whether they modulate the activity and the expression of HMGCR.

## Material and methods

**Materials.** Fetal bovine serum (FBS) and culture medium were supplied by BioWhittaker (Walkersville, MD); plasticware for cell cultures was from Falcon (Becton Dickinson, Franklin Lakes, NJ). Mevastatin was purchased from Calbiochem (San Diego, CA). Electrophoresis reagents were obtained from Bio-Rad Laboratories (Hercules, CA); the protein content of cell monolayers and lysates was assessed with the BCA kit from Sigma Chemical Co (St. Louis, MO). When not otherwise specified, all the other reagents were purchased from Sigma Chemical Co.

**Cells.** Human liver cancer HepG2 cells, human colon cancer HT29 cells and human monocytic leukemia THP-1 cells were cultured in RPMI 1640 medium supplemented with 10 % FBS, 1 % penicillin/streptomycin, 1 % L-glutamine. The rat cardiomyoblast cell line H9c2 was cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10 % FBS, 1 % penicillin/streptomycin, 1 % L-glutamine. All cell lines were maintained in humidified atmosphere at 37°C and 5 % $CO_2$.

**Cholesterol loading and measurement of the intracellular cholesterol content.** To prepare cholesterol/β-methyl-cyclodextrin (β-MCD) complexes, cholesterol was dissolved in 2-propanol/chloroform 2:1 (v/v) to give a 6.5 mM solution and added to an aqueous solution of 66.7 mM β-MCD previously heated at 80°C. During the evaporation of the solvent, cholesterol progressively associates with β-MCD forming a soluble mixture. To load cells with cholesterol, HepG2 cells were incubated with 0.67 mM β-MCD for 4 h; cells were then washed and re-suspended into fresh medium, and cholesterol/β-MCD complexes were added for 24 h at concentrations ranging from 20 to 500 μM. After this incubation time cells were lysed and the intracellular cholesterol was measured by an enzymatic colorimetric assay kit (OSR6516, Olympus System Reagent, Olympus Europe Holding GmbH, Hamburg, Germany), following the manufacturer's instructions. The absorbance was measured at 540/600 nm by an Olympus Analyzers spectrophotometer (Olympus Europe Holding GmbH), as described [13], and the results were expressed in μg cholesterol/mg cell proteins, according to a previously prepared titration curve.

**Measurement of cholesterol and ubiquinone *de novo* synthesis.** The *de novo* synthesis of cholesterol and ubiquinone was measured as previously reported [14]. Cells grown to confluence in 35-mm diameter petri dishes were incubated with 1 μCi/ml [$^3$H]acetate (3600 mCi/mmol; Amersham Bioscience, Piscataway, NJ). Cells were then washed twice with PBS and mechanically scraped in 200 μl PBS. Methanol (500 μl) and hexane (1 ml) were added to the cell suspension, which was stirred at room temperature for 1 h and then centrifuged at 2000 *g* for 5 min. The upper

phase containing hexane was transferred to a new test tube, and the lower phase was supplemented with 1 ml hexane and stirred overnight. After a 5-min centrifugation at 2000 *g*, the upper phase was added to the previous one and the solvent was allowed to evaporate at room temperature for 24 h. Cellular lipid extracts produced by this separation were re-suspended in 30 µl chloroform and then subjected to thin layer chromatography (TLC), using a 1:1 (v/v) ether/hexane solution as mobile phase. Each sample was spotted on pre-coated LK6D Whatman silica gels (Merck, Darmstadt, Germany) and allowed to run for 30 min. Solutions of 10 µg/ml cholesterol and ubiquinone were used as standards. The silica gel plates were exposed for 1 h to an iodine-saturated atmosphere, and the migrated spots were cut out. Their radioactivity was measured by liquid scintillation, using a Tri-Carb Liquid Scintillation Analyzer (PerkinElmer, Waltham, MA). Cholesterol and ubiquinone synthesis was expressed as fmol [$^3$H]cholesterol or [$^3$H]ubiquinone/$10^6$ cells, according to prepared previously calibration curves.

**Cytotoxicity assays.** After incubation under different experimental conditions (see Results), the lactate dehydrogenase (LDH) activity was measured in the extracellular medium and in the cell lysate, as previously described [15], to check the cytotoxicity of the drugs. The extracellular medium was collected and centrifuged at 12 000 *g* for 15 min to pellet cellular debris, whereas cells were washed with fresh medium, re-suspended at $1\times10^5$ cells/ml in 0.2 ml 82.3 mM triethanolamine phosphate hydrochloride (pH 7.6) and sonicated. Aliquots of supernatant (50 µl) from the extracellular medium or cell lysate (5 µl) were incubated at 37°C with 82.3 mM triethanolamine phosphate hydrochloride and 5 mM NADH. The reaction was started by adding 20 mM pyruvic acid and was followed for 6 min, measuring the absorbance at 340 nm with a Packard EL340 microplate reader (Bio-Tek Instruments, Winooski, VT). The reaction kinetics was linear throughout the time of measurement. Both intracellular and extracellular enzyme activity was expressed in µmol NADH oxidized/min per $10^6$ cells, and the extracellular LDH activity was then calculated as percentage of the total LDH activity.

Under the same experimental conditions, cells were checked for Annexin V-fluorescein isothiocyanate (FITC) positivity, as described [15]. Briefly, cells were washed twice with fresh PBS, detached with 200 µl Cell Dissociation Solution (Sigma Chemical Co) for 10 min at 37°C and re-suspended in 500 µl binding buffer (100 mM HEPES, 140 mM NaCl, 25 mM $CaCl_2$, pH 7.5). Each sample was incubated with 10 µM Annexin V-FITC for 5 min at room temperature and then the fluorescence was recorded using a FACSCalibur system (Becton Dickinson), with a 530-nm band pass filter. For each analysis 10 000 events were collected and the percentage of cells positive for Annexin V-FITC was calculated by the CellQuest software (Becton Dickinson).

**Measurement of HMGCR activity.** HMGCR activity was assayed according to [16], with the following modifications. Cells were rinsed with the lysis buffer (10 mM Tris, 100 mM NaCl, 20 mM $KH_2PO_4$, 30 mM EDTA, 1 mM EGTA, 250 mM sucrose, pH 7.5) supplemented with protease inhibitor cocktail set III (100 mM AEBSF, 80 mM aprotinin, 5 mM bestatin, 1.5 mM E-64, 2 mM leupeptin and 1 mM pepstatin; Calbiochem), 1 mM sodium orthovanadate, 1 mM NaF, 1 mM 4-(2-aminoethyl)benzenesulfonyl fluoride (PMSF), 10 mM aprotinin and 10 mM dithiothreitol (DTT). After sonication (two bursts of 10 s; Labsonic sonicator, Sartorius Stedim Biotech S.A., Aubagne Cedex, France), cell lysates were centrifuged at 13 000 *g* for 15 min at 4°C; the supernatants were subjected to ultracentrifugation at 100 000 *g* for 1 h at 4°C, using a Optima L-90K Beckman Coulter Ultracentrifuge (Beckman Coulter Inc, Fullerton, CA) to collect the microsomal fraction, which was re-suspended in 250 µl lysis buffer and stored at –80°C until the use. Microsomal protein extracts were re-suspended (12.5 µg in 25 µl) and supplemented with 10 mM DTT, 5 mM NADP and a NADPH-generating system (1.3 mM glucose 6-phosphate, 0.67 U/ml glucose-6-phosphate dehydrogenase, 33 mM $MgCl_2$). The reaction was started by adding 60 nCi [$^{14}$C]HMG-CoA (50–62 mCi/mmol, Amersham Bioscience). After a 20-min incubation at 37°C, the reaction was stopped with 25 µl 10 N HCl. The samples were stirred vigorously for 30 min at 37°C to ensure complete lactonization of mevalonic acid, centrifuged at 13 000 *g* for 2 min and separated by TLC on silica gel plates (Merck) with hexane/acetone (1:1, v/v) as mobile phase. A 1 mM solution of purified mevalonolactone was used as standard. The labeled product, [$^{14}$C]mevalonolactone, was recovered from the TLC plates and quantified by liquid scintillation. HMGCR activity was expressed as nmol HMG-CoA/mg cell proteins, according to a titration curve previously set.

**Western blot analysis.** Cells were grown to confluence in 100-mm diameter petri dishes and all the procedures for microsomal proteins extraction were performed at 4°C using ice-cold reagents. Microsomal fractions were prepared as described above, and 50 µg microsomal extracts were immunoprecipitated with

an anti-HMGCR antibody (diluted 1:100, Santa Cruz Biotechnology, Santa Cruz, CA), in the presence of 100 mM DTT and 1 mM mevalonic acid. The immunoprecipitated samples were separated by SDS-PAGE, transferred to polyvinylidene fluoride membrane sheets (Immobilon-P, Millipore, Bedford, MA) and probed with the anti-HMGCR antibody (diluted 1:500 in PBS-BSA 1 %). Microsomal proteins (10 μg) were probed with an anti-calreticulin antibody (anti-CRT; diluted 1:2000 in 1 % PBS-BSA, ABR Affinity Bioreagents, Thermo Scientific, Waltham, MA), used as a control of equal loading. After an overnight incubation at 4°C, the membranes were washed with PBS-Tween 0.1 % and subjected for 1 h to a peroxidase-conjugated secondary antibody (Bio-Rad, diluted 1:3000 in PBS-Tween with 5 % non-fat dry milk blocker). The blots were washed again with PBS-Tween, and proteins were detected by enhanced chemiluminescence (Perkin Elmer).

To detect the serine-phosphorylated HMGCR, the immunoprecipitated HMGCR was probed with a biotin-conjugated anti-phosphoserine antibody (diluted 1:1000 in 3 % TBS-BSA, Sigma Chemical Co.) for 1 h at room temperature. The membrane was washed in TBS-Tween 0.1 % and incubated for 1 h with a streptavidin- and horseradish peroxidase-conjugated polymer (diluted 1:10 000 in 3 % TBS-BSA, Sigma Chemical Co.), then washed and treated as reported above.

Immature SREBP-1 and SREBP-2 forms were detected in endoplasmic reticulum extracts. Microsomal proteins (10 μg) were subjected to SDS-PAGE and Western blotting using, respectively, an anti-SREBP-1 and an anti-SREBP-2 antibody (diluted 1:250 in 1 % PBS-BSA, Santa Cruz Biotechnology). The mature active form of SREBP-1 and SREBP-2 were measured in nuclear extracts prepared as described [15]. Cells were mechanically scraped in PBS, washed and re-suspended in lysis buffer A (10 mM HEPES, 15 mM KCl, 2 mM $MgCl_2$, 0.1 mM EDTA, 1 mM PMSF, 1 mM DTT, 10 μg/ml aprotinin, 2 μg/ml leupeptin, 0.1 % NP-40, pH 7.6). This suspension was incubated for 10 min on ice, vortexed and centrifuged for 30 s at 13 000 *g* to pellet nuclei, which were rinsed with 0.2 ml wash buffer B (25 mM HEPES, 2 M KCl, 0.1 mM EDTA, 1 mM PMSF, 1 mM DTT, 10 μg/ml aprotinin, 2 μg/ml leupeptin, pH 7.6) and incubated at 4°C for 10 min. An equal volume of buffer C (25 mM HEPES, 0.1 mM EDTA, 20 % glycerol, pH 7.6) was added and after 10 min the mix was centrifuged at 13 000 *g* for 15 min at 4°C; the supernatant was stored at –80°C until used for electrophoretic mobility shift assay (EMSA) or Western blotting. Nuclear extracts (10 μg) were probed with an anti-SREBP-1 or an anti-SREBP-2 antibody, respectively, or with an anti-proliferating cell nuclear antigen antibody (anti-PCNA; diluted 1:1000 in 1 % PBS-BSA, Santa Cruz Biotechnology), used as a control of equal loading of nuclear proteins.

**Real-time RT-PCR.** Total RNA was isolated from cells using TRIzol (Invitrogen, Milan, Italy) and 5 μg RNA were reverse-transcribed using 200 U M-MLV reverse transcriptase (Invitrogen), in the presence of 40 U/μl RNaseOUT (Invitrogen). Quantitative RT-PCR was carried out using IQ™ SYBR Green Supermix (Bio-Rad), according to the manufacturer's instructions. The same cDNA preparation was used for the quantitation of HMGCR, 3-hydroxy-3-methylglutaryl-coenzyme A synthase (HMGCS), low-density lipoprotein receptor (LDLR) and GAPDH, used as an housekeeping gene. The sequences of HMGCR, HMGCS and LDLR primers have been reported elsewhere [17]. Cycling for HMGCR, HMGCS and LDLR was: 1 cycle at 94°C for 2 min, followed by 45 cycles at 94°C for 30 s, annealing at 58°C for 30 s, extension at 72°C for 30 s. The sequences of GAPDH primers were 5'-GAAGGTGAAGGTCGGAGT-3', 5'-CATGGTG-GAATCATATTGGAA-3'. Cycling for GAPDH was: 1 cycle at 94°C for 2 min, followed by 40 cycles at 94°C for 30 s, annealing at 58°C for 30 s, extension at 72°C for 30 s. The relative quantitation of the samples was performed comparing the PCR products of each gene with the GAPDH product, using the Bio-Rad Software Gene Expression Quantitation (Bio-Rad).

**Electrophoretic mobility shift assay.** The probe containing the SRE consensus sequence derived from the HMGCR gene promoter [18] was: 5'-GATCCAGT-CAACTAGTCTCACCCCACTTCC-3' (binding site of SREBP is underlined; from Sigma Chemical Co.). The probe was labeled with 50 μCi [γ-$^{32}$P]ATP (3000 Ci/mmol, Amersham Bioscience), using a T4 polynucleotide kinase (Roche, Basel, Switzerland). Samples of nuclear extracts (10 μg), prepared as described above, were incubated for 20 min with 20 000 cpm of $^{32}$P-labeled double-stranded oligonucleotide at 4°C in a reaction mixture containing the following: 2 μl 10 μg/ml BSA, 2 μl buffer D (20 mM HEPES, 100 mM KCl, 0.5 mM EDTA, 2 mM DTT, 0.1 mM PMSF, 20 % glycerol, 0.25 % NP-40, pH 7.6), 4 μl of buffer E (100 mM HEPES, 300 mM KCl, 10 mM DTT, 100 mM PMSF, 20 % Ficoll, pH 7.6) and 2 μg poly(dI-dC) (Roche). The mixture was brought to a 25-μl final volume with water. In the supershift assay, nuclear extracts were incubated with EMSA reagents and 1 μl anti-SREBP-1X antibody (Santa Cruz Biotechnology) or anti-SREBP-2 antibody (Santa Cruz

Biotechnology) for 1 h at 4°C with gentle stirring, before adding $^{32}$P-labeled double-stranded SRE oligonucleotide for 20 min at 4°C. The DNA-protein complex was separated on a non-denaturing 4% polyacrylamide gel in TBE buffer (0.4 M Tris, 0.45 M boric acid, 0.5 M EDTA, pH 8.0). After electrophoresis, the gel was dried and autoradiographed by exposure to X-ray film for 24 h.

**SREBP-1/2 small interfering RNA transfection.** A cell suspension containing 750 000 cells/ml was chosen as the cell seeding condition having the maximal efficacy of small interfering RNA (siRNA) transfection and showing no evidence of cytotoxicity in each experimental condition, as assessed by measuring the LDH release (data not shown). Cells were plated in 35-mm diameter petri dishes and cultured in RPMI 1640 containing 10% FBS. After 24 h the cell monolayers were washed with 2 ml siRNA transfection medium (Santa Cruz Biotechnology) and incubated for 6 h with 1 ml siRNA transfection medium, containing 5 µl siRNA transfection reagent (Santa Cruz Biotechnology) and 50 pmol of either SREBP-1 or SREBP-2 siRNA (Santa Cruz Biotechnology). After incubation, 1 ml RPMI 1640 containing 1% penicillin/streptomycin and 20% FBS was added. After 24 h of incubation, cells were washed and cultured for 72 h in RPMI 1640 containing 1% penicillin/streptomycin and 10% FBS. When cells were treated with digoxin, ouabain or cholesterol/β-MCD, all the drugs were added during the last 24 h of this incubation time. To verify the siRNA efficacy, cells were subjected to differential centrifugation to collect the nuclei and the microsomal fraction, and the expression of SREBP-1 and SREBP-2 was analyzed by Western blotting as described above. In each set of experiments one dish was treated with 50 pmol Control siRNA-A (Santa Cruz Biotechnology), a scrambled non-targeting 20–25 nucleotide siRNA used as a negative control, instead of SREBPs siRNA.

**SCAP trypsinization assay.** This assay was performed according to [19], with minor modifications. The microsomal fraction (30-µg samples), obtained as described above, was incubated for 20 min at 30°C in the absence or presence of cholesterol/β-MCD, with digoxin or ouabain at different concentrations as described in Results, and then centrifuged at 13 000 *g* for 10 min at 4°C. The pellet was re-suspended in 200 µl trypsinization buffer (10 mM HEPES, 10 mM KCl, 1.5 mM $MgCl_2$, 5 mM EDTA, 5 mM EGTA, 250 mM sucrose, pH 7.5); 170 µl suspension were incubated with 0.8 µg trypsin (from bovine pancreas) for 30 min at 30 °C. The incubation was stopped by adding 100 µg tosyl-L-lysine chloromethyl ketone (TLCK), 10 µl protease inhibitor cocktail set III and 1 mM PMSF. Samples were denatured by heating at 99°C for 5 min in 5% SDS, then supplemented with 100 µl resuspension buffer (500 mM $Na_2HPO_4$, 10% v/v NP-40, 2500 U/ml PNGase-F, pH 7.4) and incubated for 4 h at 37°C. Samples were rinsed with 4 volumes of ice-cold acetone and stored for 60 min at –20°C; the solvent was evaporated and the proteins were precipitated by centrifugation at 13 000 *g* for 10 min at 4°C. Proteins were re-suspended in 15 µl Laemmli buffer, heated for 5 min at 99°C, separated by 18% SDS-PAGE and transferred to polyvinylidene fluoride membrane sheets. Blots were blocked with 5% BSA for 1 h at room temperature, then probed overnight at 4°C with a IgG-9D5 anti-SCAP antibody (diluted 1:250 in 1% PBS-BSA, Santa Cruz Biotechnology, sc-13553). The proteins detection was performed by enhanced chemiluminescence, as described.

**Statistical analysis.** All data in text and figures are provided as means ± SE. The results were analyzed by a one-way analysis of variance (ANOVA) and Tukey's test. $p<0.05$ was considered significant.

## Results

**Digoxin and ouabain increase the synthesis of cholesterol and ubiquinone.** After a 24-h incubation, both digoxin and ouabain (10 nM–1 µM) increased the synthesis of cholesterol and ubiquinone in HepG2 cells (Fig. 1A). This effect was not detectable after 6 h, but was even more evident after 48 h, when using a 10 nM concentration of cardiac glycosides (Fig. 1B). To investigate the cell viability, we measured the release of LDH activity in the extracellular medium, as an index of the drug cytotoxic effects, and performed a FACS analysis of the cells positive for Annexin V-FITC, to detect apoptosis. We did not find any significant increase in the extracellular LDH activity or in the number of Annexin V-positive cells under any of the experimental conditions (data no shown). A concentration of 10 nM digoxin or ouabain for 24 h were the minimal dose and time able to induce a significant increase of the cholesterol synthesis in HepG2 cells, without exerting any cell toxicity. For this reason we chose these experimental conditions in all subsequent experiments.

**Effect of digoxin and ouabain on cholesterol and ubiquinone synthesis is reversed by mevastatin.** The increased biosynthesis of cholesterol and ubiquinone, elicited by digoxin and ouabain, was reversed by co-incubation with the HMGCR inhibitor mevastatin,

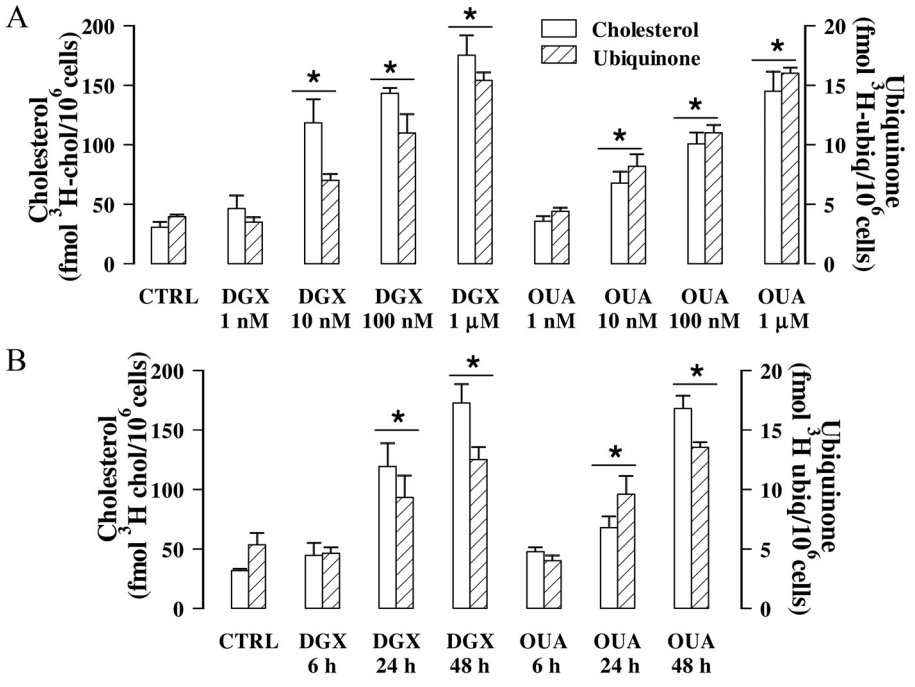


**Figure 1.** Effect of digoxin and ouabain on cholesterol and ubiquinone synthesis in HepG2 cells. (*A*) Cells were incubated for 24 h in the absence (CTRL) or presence of various concentrations (1 nM, 10 nM, 100 nM or 1 μM) of digoxin (DGX) or ouabain (OUA). (*B*) Cells were incubated for 6, 24 or 48 h in the absence (CTRL) or presence of 10 nM digoxin (DGX) or ouabain (OUA). Cells were cultured for 24 h in a medium containing [$^3$H]acetate, then the *de novo* synthesis of cholesterol (open bars) and ubiquinone (hatched bars) was measured in duplicate as described in the Materials and methods. Data are presented as means ± SE ($n$=3). *Versus* CTRL: * $p<0.05$.

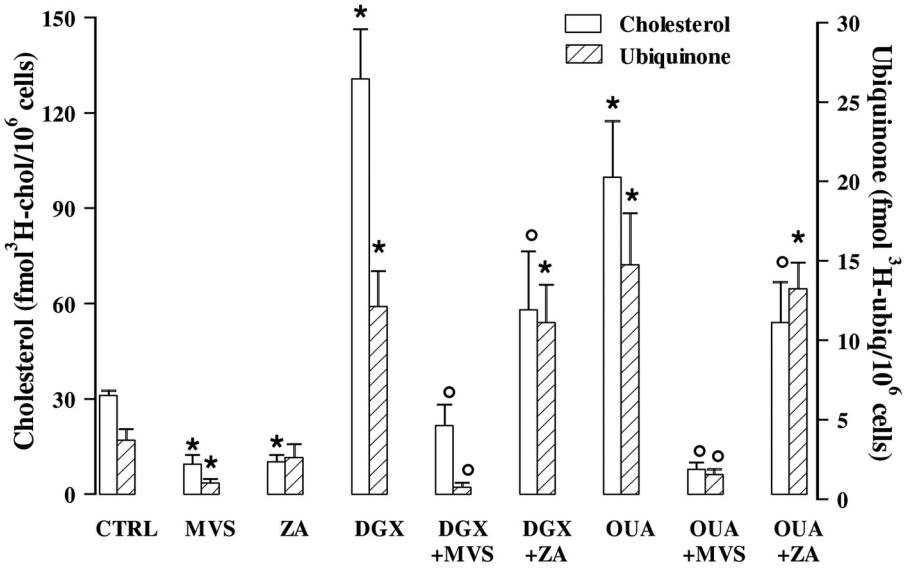


**Figure 2.** Effect of mevastatin and zaragozic acid on cholesterol and ubiquinone synthesis in HepG2 cells incubated with digoxin or ouabain. Cells were cultured for 24 h with [$^3$H]acetate in the absence (CTRL) or presence of mevastatin (10 μM, MVS), zaragozic acid (10 μM, ZA), digoxin (10 nM, DGX) and ouabain (10 nM, OUA), in different combinations. Subsequently, the synthesis of cholesterol (open bars) and ubiquinone (hatched bars) was measured in duplicate as described in the Materials and methods. Data are presented as means ± SE ($n$=4). *Versus* CTRL: * $p<0.02$; *versus* DGX or OUA: ° $p<0.05$.

which significantly reduced the cholesterol and ubiquinone amounts even when used alone (Fig. 2). In contrast to mevastatin, zaragozic acid, which is a potent inhibitor of squalene oxidase [20], decreased only the amount of cholesterol, without changing the levels of ubiquinone. When zaragozic acid was co-incubated with digoxin or ouabain, the increase in cholesterol synthesis elicited by the cardioactive glycosides was strongly inhibited, whereas the amounts of ubiquinone still remained high (Fig. 2). Taken as a whole, these results suggest that digoxin and ouabain enhance the cholesterol biosynthesis by acting at a step upstream of the squalene oxidase.

**Digoxin and ouabain increase both activity and expression of HMGCR in HepG2 cells.** We assayed the activity and expression of HMGCR in the microsomal fractions of HepG2 cells incubated for 24 h under different experimental conditions. As expected, mevastatin greatly reduced the enzyme activity compared to the control. Interestingly, digoxin and ouabain caused a twofold increase of the HMGCR activity, which was completely reversed by mevastatin

(Fig. 3A). The Western blotting analysis performed on the same extracts showed that the HMGCR expression, barely detectable in HepG2 cells, was clearly increased by mevastatin (Fig. 3B), which strongly decreased the intracellular cholesterol levels, as shown in Figure 2. Digoxin and ouabain, which enhanced the cholesterol synthesis (Fig. 2), unexpectedly increased the amount of HMGCR protein. Such an increase was not further modified by the statin (Fig. 3B).

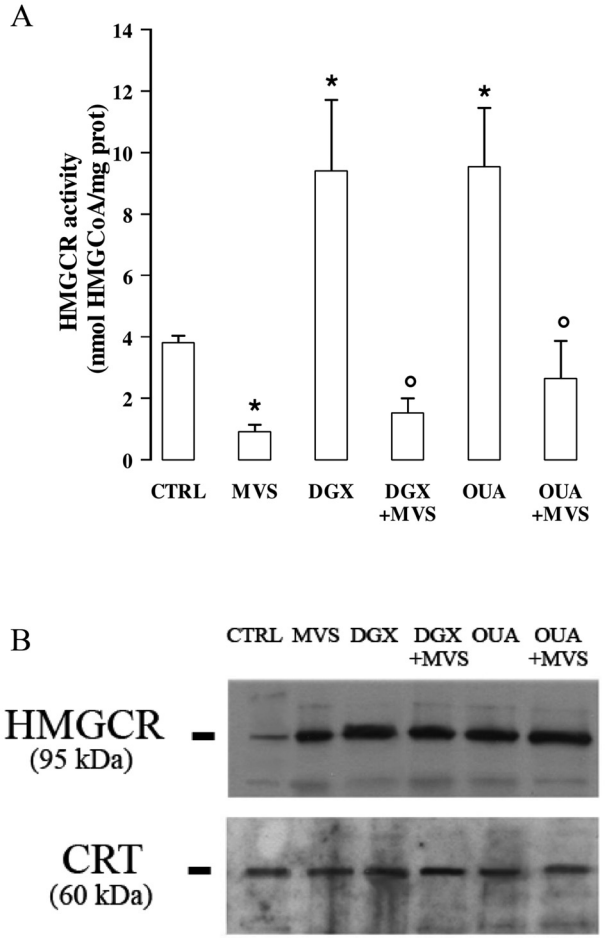


**Figure 3.** Effect of digoxin, ouabain, cholesterol and mevastatin on 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMGCR) activity and expression in HepG2 cells. Cells were grown for 24 h in the absence (CTRL) or presence of mevastatin (10 μM, MVS), digoxin (10 nM, DGX) and ouabain (10 nM, OUA), in different combinations, then lysed and centrifuged to collect the microsomal fraction (see Materials and methods), on which the following investigations were performed. (*A*) HMGCR activity was measured in duplicate (see Materials and methods). Data are presented as means ± SE ($n$=3). *Versus* CTRL: * $p<0.05$; *versus* DGX or OUA: ° $p<0.01$. (*B*) Western blotting experiments were performed using an anti-HMGCR and an anti-calreticulin (CRT) antibody, used as a control of equal protein loading (see Materials and methods). The figure is representative of three experiments with similar results.

When added to the microsomal extracts during the assay (20 min at 37°C), digoxin and ouabain did not affect either the activity or the expression of HMGCR compared to controls (data not shown).

The HMGCR activity can be inhibited by phosphorylation on serine, for instance after a glucagon stimulation [12]. A 2-h incubation of HepG2 cells with glucagon markedly reduced the enzyme activity compared to the control (Fig. 4A) and in parallel clearly induced a serine phosphorylation on HMGCR, which was absent in untreated HepG2 cells (Fig. 4B). When the cardioactive glycosides were co-incubated with glucagon, neither digoxin nor ouabain reversed the effect of glucagon on the activity (Fig. 4A) and the phosphorylation pattern of HMGCR (Fig. 4B). The expression of total HMGCR was not changed by glucagon or cardioactive glycosides after 2-h incubation (Fig. 4B). After 24-h incubation in the presence of glucagon, the activity of HMGCR was unchanged (Fig. 4A) and phosphorylation was absent (Fig. 4C). On the other hand, when glucagon was co-incubated for 24 h with digoxin or ouabain, the cardioactive glycosides-induced increase of HMGCR activity (Fig. 4A) and expression (Fig. 4C) was not modified by glucagon.

To investigate whether the increased HMGCR activity and expression elicited by glycosides was dependent on the enhanced gene transcription, we performed RT-PCR experiments. As shown in Figure 5, both digoxin and ouabain raised the mRNA levels of HMGCR after 24 h. This effect was not restricted to HMGCR; the cardioactive glycosides also increased the mRNA amounts of additional genes involved in cholesterol metabolism, such as HMGCS and LDLR (Fig. 5).

**Effect of digoxin and ouabain on HMGCR activity and expression is abolished by increasing cholesterol in HepG2 cells.** Cholesterol negatively regulates HMGCR both *via* an allosteric inhibition of the enzyme activity and by repressing the gene transcription [6]. On incubating HepG2 cells with different concentrations of cholesterol/β-MCD (20–500 μM, 24 h), the intracellular cholesterol amount increased dose dependently (Fig. 6A).

Therefore, we incubated HepG2 cells with the cardioactive glycosides (10 nM) in the presence of three cholesterol/β-MCD concentrations (20, 100 and 500 μM), and measured the HMGCR activity and expression in the microsomal extracts. The effect of cardioactive glycosides on the enzyme activity was progressively lost by increasing the intracellular amount of cholesterol: in the presence of 500 μM cholesterol/β-MCD, the HMGCR activity was completely inhibited in presence of both digoxin and

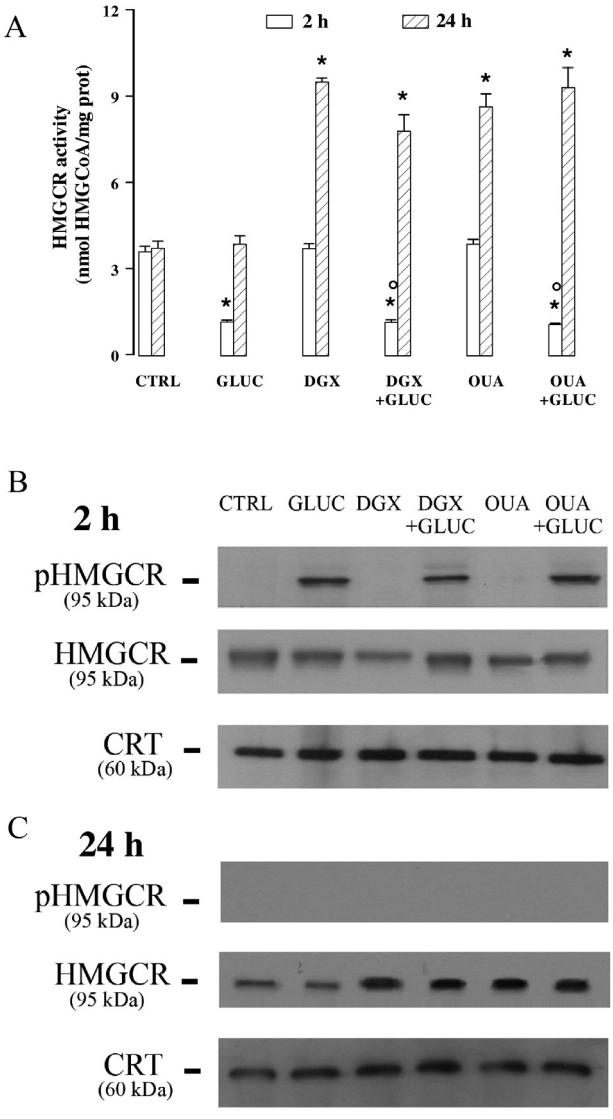


**Figure 4.** Effect of digoxin, ouabain, cholesterol and glucagon on HMGCR activity, expression and phosphorylation in HepG2 cells. Cells were incubated for 2 or 24 h in the absence (CTRL) or presence of glucagon (10 nM, GLUC), digoxin (10 nM, DGX) and ouabain (10 nM, OUA), in different combinations, then lysed and centrifuged to isolate the microsomal fraction. The following investigations were performed on the extracts. (*A*) The HMGCR activity was measured in duplicate, as described in the Materials and methods. Data are presented as means ± SE ($n$=3). *Versus* CTRL: * $p<0.001$; *versus* DGX or OUA: ° $p<0.001$. (*B, C*) Western blotting experiments were performed using an anti-HMGCR and an anti-calreticulin (CRT) antibody, used as a control of equal protein loading. To detect the HMGCR phosphorylated in serine (pHMGCR), the enzyme was immunoprecipitated with an anti-HMGCR antibody, and the sample was probed with an anti-phosphoserine antibody (see Materials and methods). The figure is representative of three experiments with similar results.

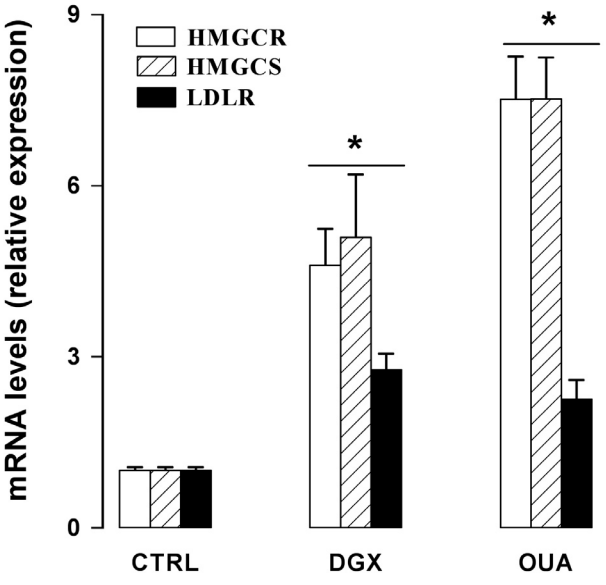


**Figure 5.** Effect of digoxin and ouabain on HMGCR, 3-hydroxy-3-methylglutaryl-coenzyme A synthase (HMGCS) and low-density lipoprotein receptor (LDLR) mRNA levels in HepG2 cells. Cells were incubated for 24 h in the absence (CTRL) or presence of digoxin (10 nM, DGX) and ouabain (10 nM, OUA), and total RNA was extracted, reverse-transcribed and subjected to RT-PCR, as indicated in the Materials and methods section. Measurements were performed in triplicate and data are presented as means ± SE ($n$=3). *Versus* CTRL: * $p<0.05$.

ouabain (Fig. 6B). A similar response pattern was observed when we measured the expression of HMGCR (Fig. 6C). These results suggested that cardioactive glycosides and cholesterol may modulate HMGCR in an opposite directions at the same regulatory step.

**Digoxin and ouabain induce SREBP2 activation by competing with cholesterol in HepG2 cells.** Since the transcription of HMGCR gene is mainly regulated by the cholesterol-sensitive SREBP1 and SREBP2 factors [6], we checked whether the cardioactive glycosides could impair the inhibition of SREBPs induced by cholesterol. We performed a set of EMSA experiments, using the SRE of the HMGCR gene promoter as a DNA probe [18]. Interestingly, we detected two DNA-protein complexes (Fig. 7A). One, with a higher molecular mass, was detectable in the nucleus in all experimental conditions and did not appear to be modulated. The complex with the lower molecular mass was not present in the nucleus of control cells, but appeared in the nucleus of cells incubated with digoxin and ouabain: cholesterol loading of the cells prevented the effect of glycosides in a dose-dependent manner (Fig. 7A). To identify the SREBP isoform(s) involved in such complexes, we performed supershift assays on the nuclear extracts of HepG2 cells treated with digoxin (10 nM, 24 h), using specific anti-SREBP antibodies. The anti-SREBP1 and anti-SREBP2 antibodies caused a gel retardation of the larger and smaller DNA-immunocomplexes, respectively, thus suggesting that the higher molecular mass complex

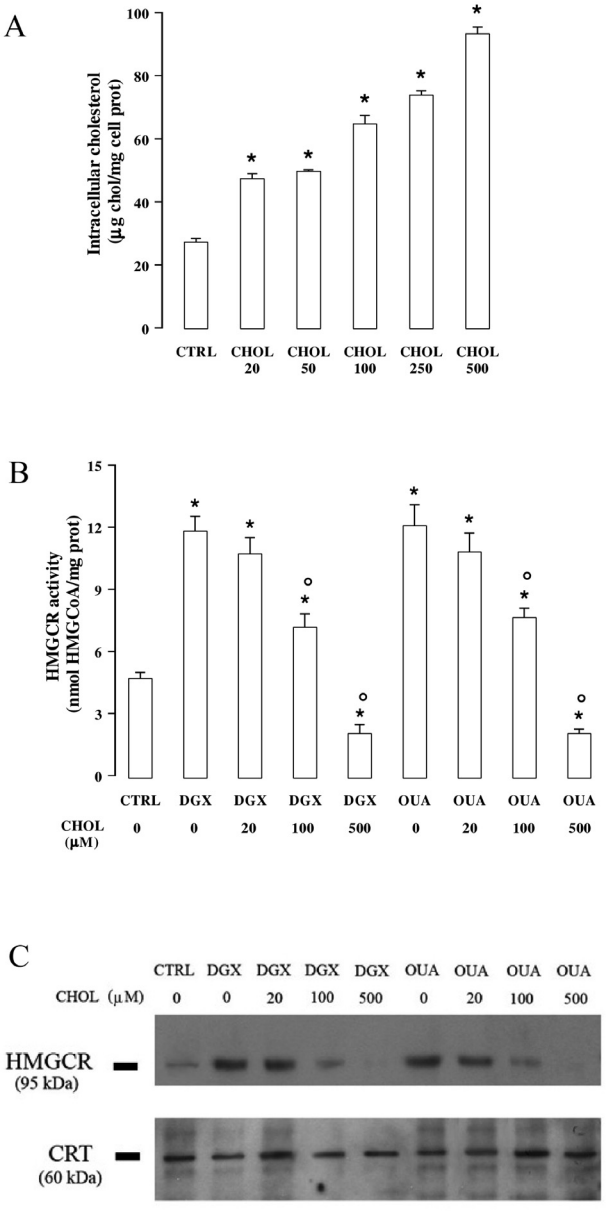


**Figure 6.** Effect of digoxin and ouabain on HMGCR activity and expression in cholesterol-loaded HepG2 cells. (*A*) Cells were grown for 24 h in the absence (CTRL) or presence (CHOL) of cholesterol/β-methyl-cyclodextrin (β-MCD) at different concentrations (20–500 µM), as described in the Materials and methods. After this incubation time, cells were lysed and the intracellular cholesterol was measured in duplicate by a spectrophotometric assay. Data are presented as means ± SE ($n$=3). *Versus* CTRL: * $p<0.001$. (*B*) Cells were cultured in the absence (CTRL) or in the presence of digoxin (10 nM, DGX) and ouabain (10 nM, OUA); when indicated, cholesterol/β-MCD at different concentrations (20–500 µM, CHOL) was added. After 24 h cells were lysed and an aliquot of the cell lysate was used to measure the intracellular cholesterol by a spectrophotometric assay (see Material and methods for details; not shown). The HMGCR activity was measured in duplicate in the microsomal fraction, collected by differential centrifugation, as described in the Materials and methods. Data are presented as means ± SE ($n$=3). *Versus* CTRL: * $p<0.02$; *versus* DGX or OUA alone: ° $p<0.005$. (*C*) Western blotting experiments were performed on aliquots of the same microsomal fractions prepared for point B, using an anti-HMGCR and an anti-calreticulin (CRT) antibody, as a control of equal protein loading. The figure is representative of three experiments with similar results.

contained SREBP1, and the lower molecular mass complex contained SREBP2 (Fig. 7A). To assess the effect of glycosides on the nuclear translocation of SREBPs, we performed a Western blot analysis of SREBP1 and SREBP2 in nuclear and cytosolic extracts of HepG2 cells. No difference in the cytosolic amounts of SREBP1 and SREBP2 was observed under any of the experimental conditions (Fig. 7B). In keeping with the EMSA results, the SREBP1 68-kDa fragment, corresponding to the mature and active form [9], was constitutively expressed in the nuclei of HepG2 cells and was non-modulatable, whereas the mature active SREBP2 form was absent in the nuclear extracts of untreated HepG2 cells and was induced by digoxin and ouabain (Fig. 7C). The increase of nuclear SREBP2 elicited by the cardioactive glycosides was reversed by incubating the cells with increasing amounts of cholesterol (Fig. 7C).

**Effect of digoxin and ouabain on HMGCR activity and expression is abolished in SREBP2-silenced HepG2 cells.** To confirm that SREBP2 plays the major role in the modulation of HMGCR expression by cardioactive glycosides, we silenced either SREBP1 or SREBP2 in HepG2 cells. First, we checked the efficacy of the silencing protocol in cells stimulated with digoxin, an experimental condition which increased nuclear SREBP2 expression in HepG2 cells (Fig. 7C): after the incubation with the specific siRNA for each SREBP isoform, the expres-

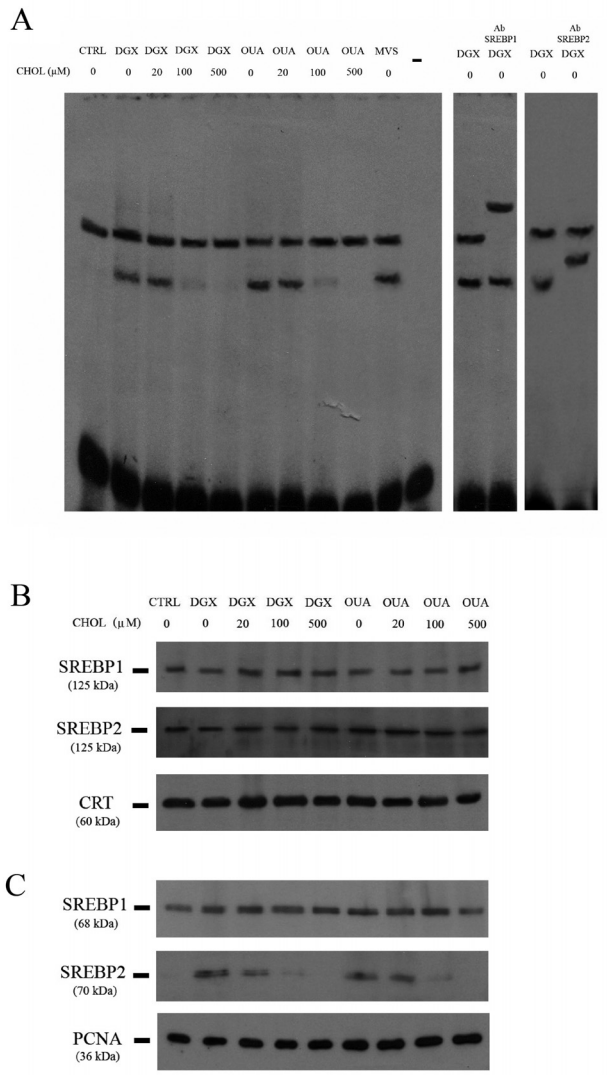


**Figure 7.** Effect of digoxin and ouabain on sterol regulatory element binding protein (SREBP)1 and SREBP2 activity and expression in cholesterol-loaded HepG2 cells. Cells were grown for 24 h in the absence (CTRL) or presence of digoxin (10 nM, DGX) and ouabain (10 nM, OUA); when indicated, cholesterol/β-MCD at different concentrations (20–500 μM, CHOL) was added. After this incubation time, cells were lysed and an aliquot of the cell lysate was used to measure the intracellular cholesterol spectrophotometrically (see Material and methods; not shown). The following investigations were performed on the cell lysates. (*A*) The EMSA detection of SREBPs binding to the HMGCR gene promoter was performed on nuclear extracts (see Materials and methods). This figure is representative of three experiments with similar results. In each experiment one lane was loaded with bidistilled water (-) in place of cellular extracts. Mevastatin (10 μM for 24 h, MVS) was added as a positive control of SREBPs activation. In the experimental points marked "Ab SREBP1"or "Ab SREBP2" supershift assays were performed in cells incubated with digoxin (10 nM for 24 h), as described in the Materials and methods. (*B*) The cell lysate was subjected to differential centrifugation. The expression of SREBP1, SREBP2 and CRT (used as a control of equal protein loading), was checked in the microsomal fraction by Western blotting (see Materials and methods). The figure is representative of three experiments with similar results. (*C*) Western blotting detection of SREBP1 and SREBP2, performed on nuclear cellular extracts, as described in the Materials and methods. Proliferating cell nuclear antigen (PCNA) expression was used as a control of equal nuclear protein loading. The figure is representative of three experiments with similar results.

sion of SREBP1 and SREBP2, respectively, was strongly reduced in both microsomal and nuclear fractions, whereas the non-targeting siRNA used as a control of the silencing specificity had no effect (Fig. 8A–D). We then measured the expression and activity of HMGCR in HepG2 cells silenced for either SREBP1 or SREBP2 and grown in the absence or presence of digoxin, ouabain and varying amounts of cholesterol (Fig. 9). In SREBP1-knocked-down cells, HMGCR was hardly detectable in the control cells, while it clearly appeared after incubation with digoxin and ouabain, an effect which was dose-dependently prevented by the co-incubation of cardioactive glycosides with increasing concentrations of cholesterol (Fig. 9A). On the other hand, after SREBP2 silencing, we found a weak, non-modulated, HMGCR expres-

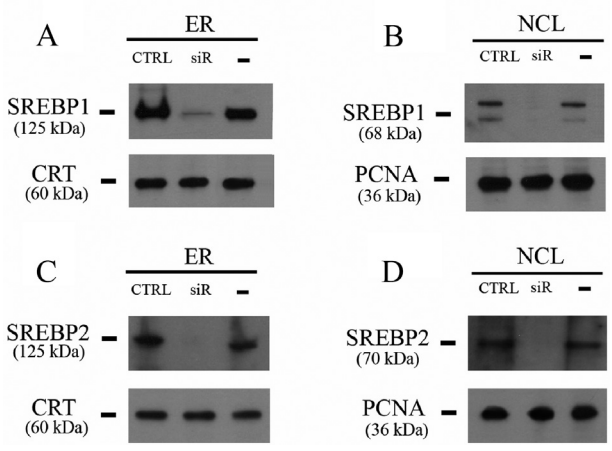


**Figure 8.** Efficacy of SREBP1 and SREBP2 silencing in HepG2 cells. HepG2 cells were cultured for 72 h in RPMI medium alone (CTRL) or in the same medium containing specific siRNA for SREBP1 or SREBP 2 (siR) or a scrambled non-targeting 20-25-nucleotide siRNA, designed as a negative control (-). Under all the experimental conditions 10 nM digoxin was added during the last 24 h. Cells were then lysed and centrifuged to collect microsomal (ER) and nuclear (NCL) extracts, as described in the Materials and methods. (*A, B*) Western blotting detection of residual SREBP1 expression in cells subjected to SREBP1 silencing. (*C, D*) Western blotting detection of residual SREBP2 expression in cells subjected to SREBP2 silencing. The expression of CRT and of the PCNA was checked as a control of equal loading of proteins from microsomal and nuclear fractions, respectively. Each figure is representative of three experiments with similar results.

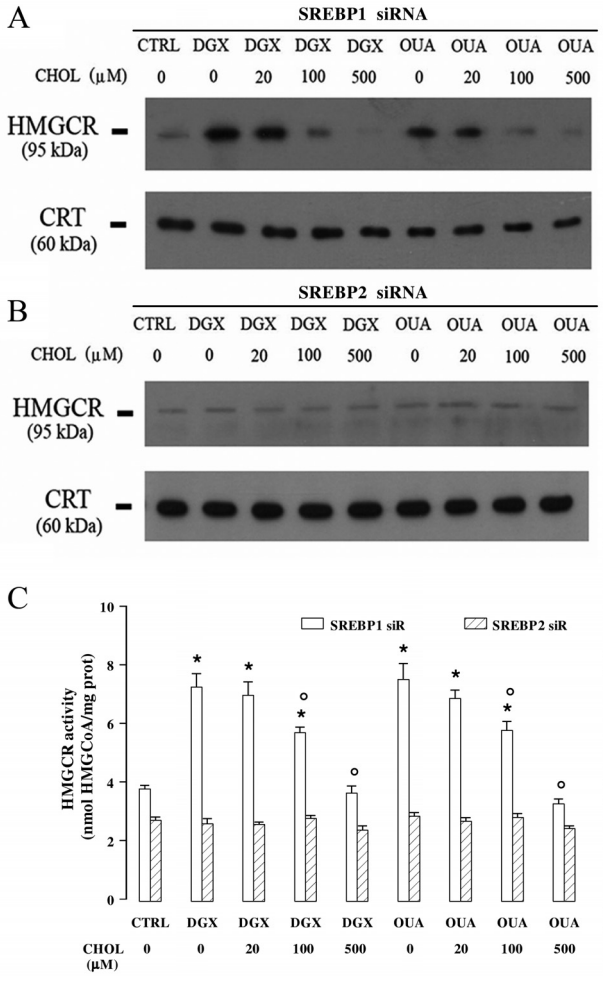


**Figure 9.** Effect of digoxin and ouabain on HMGCR activity and expression in cholesterol-loaded and SREBP1/2-silenced HepG2 cells. Cells were silenced for SREBP1 or SREBP2 protein, as described in the Materials and methods, in the absence (CTRL) or presence of digoxin (10 nM, DGX) and ouabain (10 nM, OUA); when indicated, cholesterol/β-MCD at different concentrations (20–500 μM, CHOL) was added. Cells were then lysed and an aliquot of the cell lysate was used to measure spectrophotometrically the intracellular cholesterol (see Material and methods; not shown). In the microsomal fraction, collected by differential centrifugation, the following investigations were performed. (*A, B*) Western blotting experiments on SREBP1-silenced (*A*) or SREBP2-silenced (*B*) cells were performed using an anti-HMGCR and an anti-CRT antibody (control for equal protein loading). The figure is representative of three experiments with similar results. (*C*) HMGCR activity was measured in duplicate (see Materials and methods). Data are presented as means ± SE ($n$=3). *Versus* CTRL: * $p<0.01$; *versus* DGX or OUA alone: ° $p<0.05$.

sion in all experimental conditions (Fig. 9B). The expression of calreticulin, the product of a housekeeping gene unrelated to SREBP1/2 protein, was not changed by the transfection procedure (Fig. 9A, B). The activity of HMGCR in the same extracts varied accordingly (Fig. 9C): in SREBP1- or SREBP2-silenced control cells the enzyme activity was similar but slightly lower than the activity observed in non-silenced cells (Figs. 3A, 4A, 6B). When SREBP1 was knocked-down, HepG2 cells still exhibited an increasing HMGCR activity when incubated with digoxin and ouabain, which was blunted by co-incubation with cholesterol; in contrast, in SREBP2-silenced cells, the HMGCR activity was not modulated by cardioactive glycosides or cholesterol, showing values superimposable to those measured in control cells (Fig. 9C).

**Digoxin and ouabain interfere with the conformational change of SCAP elicited by cholesterol.** In sterol-depleted cells, the SREBPs mobilization from the endoplasmic reticulum is enhanced by the tight binding with SCAP, which plays a dual role as an escort protein to the Golgi and sterol sensor [19]. The SCAP conformational change in response to cholesterol was assessed by a trypsin cleavage assay, which detects the accessibility of specific arginine residues to trypsinization, which is favored when SCAP is bound to cholesterol. In sterol-depleted cells or in the presence of sterols other than cholesterol, SCAP has a conformation that makes it refractory to trypsin cleavage [19]. The microsomal extracts of HepG2 cells were incubated with either digoxin or ouabain, in the absence or presence of increasing amounts of cholesterol (Fig. 10A). None of the SCAP fragments resulting from trypsin digestion was detectable in

controls and extracts incubated with digoxin or ouabain alone. In contrast, a fragment of about 26 kDa, an index of the trypsin digestion of SCAP [19], became progressively more evident in microsomal extracts loaded with cholesterol (Fig. 10A). In a second set of experiments, we incubated the microsomal extracts with a constant concentration (10 μM) of cholesterol, varying the digoxin and ouabain concentration from 0.01 to 1 μM (Fig. 10B). The 26-kDa SCAP cleavage product was the only band detectable in the presence of cholesterol, whereas it progressively disappeared on raising the amounts of cardioactive glycosides. In the presence of 1 μM digoxin or ouabain and 10 μM cholesterol, no trypsin-digestion product of SCAP was detectable (Fig. 10B).

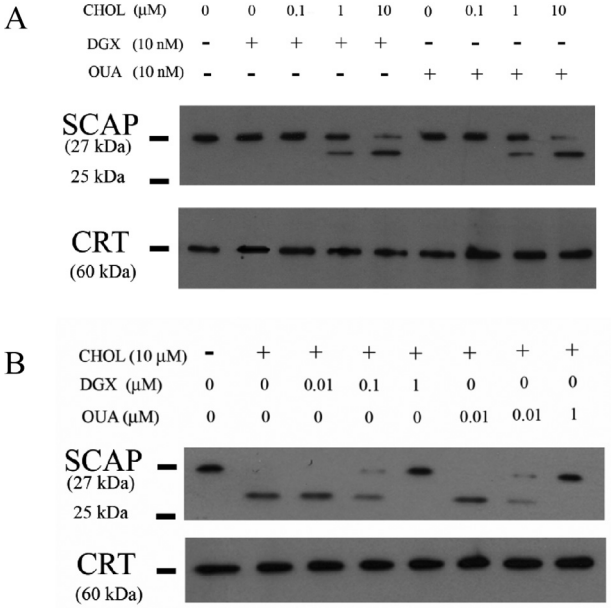


**Figure 10.** Effect of digoxin and ouabain on cholesterol-elicited SREBP cleavage-activating protein (SCAP) trypsinization in HepG2 cells. (*A*) A 30 μg microsomal fraction was incubated at 30°C for 20 min in the absence (-) or presence of cholesterol/β-MCD (0.1–10 μM, CHOL), 10 nM digoxin (DGX) and 10 nM ouabain (OUA), in different combinations. (*B*) 30 μg of microsomal fraction were incubated at 30°C for 20 min in the absence (-) or presence of 10 μM cholesterol/β-MCD (CHOL), digoxin (0.01–1 μM, DGX) and ouabain (0.01–1 μM, OUA), in different combinations. Subsequently, the trypsinization assay of SCAP protein was performed and the SCAP fragments obtained after the cleavage were separated by SDS-PAGE and identified by Western blotting (see Materials and methods). The Western blotting analysis of CRT was used as a control of equal protein loading. The figure is representative of three experiments with similar results.

## Discussion

Cardioactive glycosides, such as digoxin and ouabain, by inhibiting the $Na^+/K^+$-ATPase pump and increasing the intracellular $Ca^{2+}$ levels, exert a positive inotropic effect on cardiac muscle [1]. Because of its toxicity ouabain is used mainly in experimental studies [21], whereas digoxin is the most commonly glycoside used in cardiological therapy [22, 23], with a therapeutic range between 0.6 and 2.5 μM in blood [24, 25]. Recently, a family of endogenous ouabain-like factors released by adrenal glands has been discovered in mammalian tissues: they may play a role in maintaining the homeostasis of water and mineral salts and controlling the blood pressure [26]. Their plasma concentration is in the picomolar range and their effect seems to be independent from the inhibition of $Na^+/K^+$-ATPase [27].

It has been reported that cardioactive glycosides may exert effects unrelated to the inhibition of $Na^+/K^+$-ATPase, but dependent on their steroid structure. Digoxin and ouabain affect the synthesis of steroid hormones, such as aldosterone [3] and corticosterone [5], in human adrenal glands, and chronic administration of ouabain stimulated the synthesis of aldosterone and corticosterone in rat adrenal glands [4].

Since the synthesis of cholesterol, the precursor of steroid hormones, is regulated by sterols, we wondered whether digoxin and ouabain may have an effect on the intracellular production of cholesterol. We focused our investigation on human liver HepG2 cells, since liver is a major site of cholesterol synthesis. In these cells we observed that 10 nM digoxin and ouabain for 24 h significantly increase the production of cholesterol and ubiquinone (which derives its isoprenoid lateral chain from the farnesyl-pyrophosphate intermediate of the cholesterol synthesis).

In the presence of mevastatin and zaragozic acid, which inhibit HMGCR and squalene oxidase, respectively, the effect of the two glycosides on cholesterol production was reversed, but only mevastatin prevented the effect of glycosides on the level of ubiquinone. These results suggest that digoxin and ouabain activate a step upstream of the squalene oxidase reaction. We hypothesized that cardioactive glycosides might modulate the rate-limiting enzyme of cholesterol and ubiquinone biosynthesis, HMGCR, which may be finely regulated at the level of gene transcription, mRNA translation, and protein degradation and *via* the covalent or allosteric modulation of the enzyme activity [6, 7, 9].

Both digoxin and ouabain increased the HMGCR activity and expression in the microsomal fraction. In contrast, mevastatin reversed the glycoside effect on the HMGCR activity, but not on its expression. Indeed, when cells are depleted of sterols, for instance in the presence of statins, the amount of HMGCR is increased, because the negative feedback exerted by cholesterol on the HMGCR gene transcription is

abolished [6]. Mevastatin did not further enhance the effect of glycosides, suggesting that the drugs may *per se* exert the maximal stimulation on HMGCR.

When added directly to the microsomal extracts during the HMGCR assay, digoxin and ouabain did not increase the enzyme activity, thus excluding they act as allosteric modulators of HMGCR. The HMGCR activity can be inhibited by phosphorylation on serine, for instance by glucagon [12]. After a 2-h incubation period glucagon induced the HMGCR phosphorylation and reduced the enzyme activity; at this time point the cardioactive glycosides had no effect on HMGCR expression and activity. After 24-h incubation, in the presence of glucagon, the activity of HMGCR was unchanged and the phosphorylation was absent. On the other hand, when glucagon was co-incubated for 24 h with digoxin or ouabain, the cardioactive glycoside-induced increase of HMGCR activity and expression was not modified by glucagon. The mRNA levels of HMGCR, as well as of other genes involved in cholesterol metabolism, were increased by digoxin and ouabain after 24 h. In the light of our results, we hypothesize that the enhanced expression of HMGCR elicited by the glycosides was not due to a change in the phosphorylation status of the enzyme, but rather to an increased gene transcription.

Since digoxin and ouabain produce the same effects of a sterol depletion, we hypothesized that cardioactive glycosides might affect the cholesterol-sensing mechanism involved in the negative feed-back inhibition of HMGCR. When cells are loaded with sterols, HMGCR activity and expression are repressed [6], an experimental evidence that we confirmed in HepG2 cells. Moreover, digoxin and ouabain progressively lost their ability to enhance HMGCR activity and expression when cells were loaded with increasing amounts of cholesterol. These results suggest a competitive mechanism between cholesterol and digoxin/ouabain in the cholesterol-sensing machinery of HepG2 cells.

The transcription of HMGCR gene, as well as of HMGCS and LDLR, is tightly regulated by the SREBPs, a family of transcription factors that are located in the endoplasmic reticulum as inactive precursors; when the sterols concentration within the cell is low, SREBPs are translocated to the Golgi, and their N-terminal domain is proteolytically released and reaches the nucleus, where it induces the transcription of the target genes by binding the specific SRE sequence in the promoter. When the cholesterol cell content rises, SREBPs change their conformation and cannot be released from the endoplasmic reticulum [9]. In mammalian tissues three SREBP isoforms have been identified, SREBP1a, SREBP1c and SREBP2. SREBP1a is a potent activator of all SRE-responsive genes, whereas the role of SREBP1c and SREBP2 is more restricted [8, 28]: SREBP1c preferentially enhances the transcription of genes involved in fatty acid synthesis, while SREBP2 controls genes of cholesterol synthesis [29]. With EMSA, supershift assays and Western blotting analysis we detected two proteins recognizing the same SRE sequence on the HMGCR promoter: one, identified as SREBP1, was constitutively present in the nucleus and was non-modulated, whereas the nuclear expression of SREBP2 was absent in untreated cells, increased by digoxin or ouabain and reversed by the addition of increasing concentrations of cholesterol, showing the same pattern observed in the regulation of HMGCR. We hypothesize that SREBP1 is responsible for the basal expression and activity of HMGCR, whereas SREBP2 accounts for the regulation of HMGCR activity through the cellular cholesterol levels. The effect of digoxin, ouabain and cholesterol on the activity and expression of HMGCR was not modified by the silencing of SREBP1, in comparison to non-silenced cells. In contrast, in cells silenced for SREBP-2, neither the cardioactive glycosides nor cholesterol were able to modulate HMGCR expression and activity, which remained at the same level under all experimental conditions. These results confirm that digoxin and ouabain enhance HMGCR activity and expression by activating SREBP2.

Although SREBP2 plays a central role in cholesterol homeostasis [19], the HMGCR gene can be regulated by many other proteins, such as the cyclic AMP-responding element binding protein [30], and the hypoxia-inducible factor-1α [31]. Therefore, we cannot exclude that at least part of the effect of digoxin and ouabain on HMGCR might be mediated by these mechanisms.

In sterol-depleted cells, SREBPs usually bind to SCAP, a microsomal protein that acts both as escort and sterol-sensor protein. The SCAP/SREBP complex reaches the Golgi within budding vesicles, where SREBPs are activated. Cholesterol binds to the sterol-sensitive domain of SCAP and triggers a conformational change, which prevents the SCAP/SREBP complex from leaving the endoplasmic reticulum [8, 9] and unmasks a site of cleavage for trypsin on SCAP [19]. In our experiments the SCAP trypsinization assays suggest that digoxin and ouabain compete with cholesterol for binding to the SCAP sterol-sensitive domain, and have an affinity ten times higher than cholesterol for SCAP. It has been reported that single nucleotide polymorphisms in the SCAP gene could produce sterol-resistant mutants, capable of binding and activating SREBP constitutively [19]. Other

compounds having a sterol-like structure could compete with cholesterol for the binding to SCAP: changes of chemical groups on the sterol nucleus and of their spatial orientation may be critical in making such compounds SCAP activators or repressors [19].

In summary, our results suggest that digoxin and ouabain, owing to their steroid structure, could activate the SREBP2/SCAP complex in competition with cholesterol, enhance the expression and activity of HMGCR, and increase the synthesis of cholesterol and isoprenoids like ubiquinone. The effects of cardiac glycosides on cholesterol and isoprenoid biosynthesis is not cell specific, because we also detected effects similar to those observed in HepG2 cells in human colon cancer HT29 cells, in human monocytic leukemia THP-1 cells and in rat cardiomyoblast H9c2 cells (data not shown). To our knowledge, this is the first study describing the ability of cardiac glycosides digoxin and ouabain to modulate cholesterol biosynthesis. These findings might have physiopathological consequences. For instance, it is known that different levels of cholesterol in plasma membranes may modify the activity of $Na^+/K^+$-ATPase and its affinity for inhibitors [32]. Thus, the therapeutic efficacy of the glycosides could be influenced by their stimulating effects on intracellular cholesterol synthesis. Moreover, changes of cholesterol levels, *e.g.*, caused by dyslipidemic states or statins therapy, might produce a different response to cardioactive glycosides. Since dyslipidemia and heart failure are frequently associated [33], the potential interference between digoxin and cholesterol should be taken into consideration.

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