Signaling through cholesterol esterification: a new pathway for the cholecystokinin 2 receptor involved in cell growth and invasion

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Abstract   Several studies indicate that cholesterol esterification is deregulated in cancers. The present study aimed to characterize the role of cholesterol esterification in proliferation and invasion of two tumor cells expressing an activated cholecystokinin 2 receptor (CCK2R). A significant increase in cholesterol esterification and activity of Acyl-CoA:cholesterol acyltransferase (ACAT) was measured in tumor cells expressing a constitutively activated oncogenic mutant of the CCK2R (CCK2R-E151A cells) compared with nontumor cells expressing the wild-type CCK2R (CCK2R-WT cells). Inhibition of cholesteryl ester formation and ACAT activity by Sah58-035, an inhibitor of ACAT, decreased by 34% and 73% CCK2R-E151A cell growth and invasion. Sustained activation of CCK2R-WT cells by gastrin increased cholesteryl ester production while addition of cholesteryl oleate to the culture medium of CCK2R-WT cells increased cell proliferation and invasion to a level close to that of CCK2R-E151A cells. In U87 glioma cells, a model of autocrine growth stimulation of the CCK2R, inhibition of cholesterol esterification and ACAT activity by Sah58-035 and two selective antagonists of the CCK2R significantly reduced cell proliferation and invasion. In both models, cholesteryl ester formation was found dependent on protein kinase zeta/ extracellular signal-related kinase 1/2 (PKCζ/ERK1/2) activation.   These results show that signaling through ACAT/cholesterol esterification is a novel pathway for the CCK2R that contributes to tumor cell proliferation and invasion.—Paillasse, M. R., P. de Medina., G. Amouroux, L. Mhamdi, M. Poirot, and S. Silvente-Poirot. Signaling through cholesterol esterification: a new pathway for the cholecystokinin 2 receptor involved in cell growth and invasion. *J. Lipid Res.* 2009. **50:** **2203–2211.**

Supplementary key words   cholesteryl ester • acyl-CoA:cholesterol acyltransferase • constitutively active mutant • autocrine stimulation • G protein coupled receptor • gastrin receptor • glioma • cancer

This article is available online at http://www.jlr.org

The cholecystokinin 2 receptor (CCK2R) is a G protein-coupled receptor that mediates important physiological functions by binding cholecystokinin and gastrin peptides (1, 2). The CCK2R regulates the growth of normal and neoplastic gastrointestinal mucosal cells and the growth of different cells outside the digestive system (3–11). The growth-promoting action of the CCK2R has led to extensive investigations of its role in carcinogenesis and its expression in cancer. The expression of the CCK2R has been reported on malignant adenocarcinomas arising within all areas of the gastrointestinal tract mucosa, hepatomas, and colorectal liver metastases, as well as in different cancers of neuroendocrine origin (8, 12–15). CCK2R expression may also be coupled with the coexpression of gastrin and CCK in different human tumors and cancer cell lines. A permanent activation of the CCK2R by a gastrin/CCK-CCK2R autocrine loop has been reported to promote cell growth in different tumor cells (4, 14–20).

We have developed an experimental model of permanent activation of the CCK2R by expressing a constitutively active mutant of the CCK2R in NIH-3T3 cells resulting from the mutation of the Glu151 residue to an Ala (CCK2R-E151A mutant). The CCK2R-E151A mutant induced increased cell proliferation and invasion as well as the formation of tumors in mice, while no such effects were observed when the CCK2R wild-type was expressed in NIH-3T3 cells, showing a link between the constitutive activation of the CCK2R and tumor formation (21). Although the proliferative effects of the CCK2R after its activation are well recognized, the molecular and intracellular mechanisms

by which the CCK2R modulates cell growth have not been fully elucidated. Previous studies have reported that the stimulation of the CCK2R by its agonist ligands activates various signal transduction pathways implicated in cell proliferation, including phospholipase C, mitogen-activated protein kinases, expression of early growth-responsive genes, and activation of ornithine decarboxylase (ODC) (6). More recently, gastrin has been shown to activate cell proliferation in colorectal tumors by a mechanism involving the phosphorylation and degradation of the nuclear receptor peroxisome proliferator-activated receptor γ, a transcriptional factor reported to lower cholesterol metabolism when activated by agonist ligands (22). A link between CCK2R activation and the regulation of cholesterol metabolism has recently been reported in neuronal cells. Indeed, PC12 cells transfected with the CCK2R regulated a set of genes involved in cholesterol metabolism in response to CCK (23). However, to date, no studies have implicated the cholesterol metabolism in the growth-promoting effects of the CCK2R in tumor cells. Cholesterol is a crucial component of cell membranes that plays an important role in the organization of lipid bilayers and is essential for membrane biogenesis and cell proliferation. In addition, cholesterol has been shown to modulate the functions of G protein coupled-receptors and to participate in several membrane trafficking and transmembrane signaling processes (24–26). Cholesterol synthesis is normally tightly regulated to maintain the appropriate cholesterol level in cells. Cholesterol esterification by acyl-CoA:cholesterol acyltransferase-1 and -2 (ACAT1 and ACAT2) is a mechanism used by cells to prevent the accumulation of free cholesterol and its conversion to oxysterols (26, 27). Increased production of cholesteryl esters, ACAT expression and activation has been measured in different human tumors compared with normal tissue. In addition, a greater capacity to esterify and accumulate cholesterol in tumor cells has been associated with a higher growth rate, suggesting a link between cholesteryl ester production and cell proliferation (28–32).

The present study was undertaken to characterize the pattern of cholesterol esterification in tumor cells expressing an activated CCK2R and its role in cell proliferation and invasion mediated by this receptor. For this purpose, we used the tumor model of permanent activation of the CCK2R that we have engineered versus that of the non-tumor cells expressing the wild-type CCK2R (21) as well as U87, a human glioma cell line expressing an activated CCK2R in which proliferation has been shown to be stimulated by CCK2R agonists in an autocrine manner (20).

MATERIALS AND METHODS

Materials

Sah58-035 (PRK 058035) was provided by Novartis Institutes for BioMedical Research (Switzerland). L365260 and YM022 were kindly provided by Dr. A. Bado (INSERM U77, Paris 7, Paris, France) and the dominant negative (T410A) PKCζ plasmid (dn PKCz) by Pr. Ferdinando Auricchio (University of Naples, Italy). Inhibitors were from Calbiochem. Gastrin was from Neosystem.

Cholesteryl oleate (CO) was from Sigma-Aldrich and its purity was verified by TLC. [14C]cholesterol (58 Ci/mol) and [14C]cholesteryl oleate (58 Ci/mol) (1 Ci = 37GBq) were purchased from General Electric Healthcare (Little Chalfont, Bukhinghamshire, UK). Solvents were from VWR. Silica gel TLC plates (60 Å pores) were from Flüka. The antibodies were from Cell Signaling. Matrigel® was from Becton Dickinson.

Cell culture

All cell lines were grown in DMEM supplemented with 10% fetal calf serum (FCS) and 2 mM L-glutamine. NIH-3T3 clones stably expressing CCK2R-WT, CCK2R-E151A, and empty vector were obtained previously (21). U87-MG cells were from LGC, UK. Cells were split when they were 80% confluent. In all experiments, cells were counted using a Coulter counter (Beckman-Coulter).

Cholesterol esterification analysis

Cells were seeded in 6-well plates (40,000 cells/well) in DMEM with 10% FCS. Two days after seeding, cells were simultaneously treated for 24 h with 14C-cholesterol (0.1 μCi/μl–2 μl/dish) and with the indicated inhibitors or vehicle, or with 10 nM gastrin or vehicle every h over 16 h. After incubation, cells were washed, scraped, and neutral lipids were extracted with chloroform-methanol (2:1, v/v) as in (33). The chloroform phase containing the lipids was evaporated to dryness under nitrogen and the residues were dissolved in 50 μl of ethanol at 4°C. Samples were spotted on Fluka 20 × 20 silica gel plates previously heated 1 h at 100°C and developed using n-hexane-diethyl ether-acetic acid (70:30:1, v/v/v). The radioactive metabolites were identified and quantified on TLC plates either by using a GP storage phosphor screen (GE Healthcare) and a phosphorimager (STORM 840, GE Healthcare; the peak integration was obtained by using ImageQuant 5.2 software) or by liquid scintillation counting of the cholesteryl ester region. The positions of cholesterol and cholesteryl esters were determined using 14C-radiolabeled cholesterol and CO standards. The Rf for cholesterol and CO were 0.20 and 0.85, respectively.

Transfection assay

Cells were seeded in 6-well plates (40,000 cells/well) in DMEM with 10% FCS. One day after seeding, cells were transfected with 2 μg mock or dominant negative-PKC ζ with Fugene 6® transfection reagent (Roche) in a 1:6 (v/v) ratio. One day after transfection, cells were incubated with 14C-cholesterol (0.1 μCi/μl – 2 μl/dish) in DMEM with 10% FCS for 24 h. Cholesterol esterification was then determined as described above.

ACAT activity assay on cell lysates

Cells were seeded in 140 mm diameter dishes (450,000 cells/dish) in DMEM with 10% FCS. Two days after, the dishes were treated for 24 h with Sah 58-035 or with the vehicle. Cells were then pelleted and disrupted in a manual ground-glass homogenizer in 150 μl of 10 mM-potassium phosphate (pH 7.4)/1 mM-dithiothreitol/1 mM-EDTA and protein concentrations were determined as in (34). Assays for ACAT activity were performed by measuring the formation of cholesteryl [14C]oleate as reported in (35). Briefly, 280 μg of proteins in 250 μl 0.1 M potassium phosphate buffer (pH 7.4) containing 5% volume dimethylformamide and 9 nmol of fatty acid-free BSA were incubated with [14C]Oleyl CoA (100 μM, 0.25 μCi) for 5 min at 37°C. The reaction was terminated by adding 3 ml of chloroform-methanol (2:1) and 500 μl of 0.04 N HCl. After shaking, the lower phase was taken and reduced to dryness under nitrogen. Lipids were analyzed by TLC as described above.

Quantitative real-time PCR

Cells were grown in 100 mm dishes in DMEM with 10% FCS until they were 80% confluent. Total RNA was extracted using the QIAmp RNA kit (Qiagen). RNA integrity was measured using an Agilent 2100 Bioanalyzer. The DNA strand synthesis was carried out using 1 μg of DNase-treated RNA with the iScript™ cDNA Synthesis kit (Bio-Rad) following the protocol provided by the manufacturer. Real-time PCR was carried out using the icycler iQ™ Real Time Detection system (Bio-Rad). Amplification reactions were done with the iQ™SYBR Green Supermix (Bio-Rad). The forward and reverse primers used at 900 nM were, respectively: 5′-TACCGAGACAACTACCCAAGG-3′ and 5′-AGGACACGAGCACTGAAGG-3′ for ACAT1; 5′-GCTCT-GCTGCTCTCCATC-3′ and 5′-GTTCCAGGTGCGGTAGTAG-3′ for ACAT-2; 5′-ACTTCGTGCAAGAAATGCTGAA-3′ and 5′-GCAG-TTGTCCGTGGCTCTCT-3′ for Tata Box binding protein (TBP). Amplification was carried out as follows: 95°C for 3 min, followed by 40 cycles at 95°C for 15 s, 60°C for 1 min (iCycler, Bio-Rad). Levels of ACAT1 and ACAT2 were normalized to TBP. Threshold cycle (Ct) values were generated by the iCycler iQ software (Bio-Rad).

Western blotting analysis

Cells were seeded in 100 mm diameter dishes (500,000 cells/dish) in DMEM with 10% FCS. After 2 days, cells were incubated for 24 h with the indicated inhibitors, then stimulated or not with 10 nM gastrin for 10 min, washed, and incubated with lysis buffer [30 mM HEPES (pH 7.5), 1%, Triton ×100 10% glycerol, 10 mM NaCl, 25 mM NaF, 5 mM MgCl$_2$, 1 mM EDTA] in the presence of a mixture of protease inhibitors (Sigma-Aldrich) for 45 min at 4°C and centrifuged at 12,000 × g for 10 min at 4°C. The proteins were separated on 10% SDS-PAGE gels, electrotransferred onto polyvinylidene difluoride membranes, and incubated overnight at 4°C with the rabbit anti-phospho ERK1/2 (Thr202/Tyr204) (1:1,000) or the rabbit anti-ERK2 (1:1,000). After saturation, the membranes were incubated 1 h at 37°C with the rabbit anti-Hrp (1/1000). Visualization was achieved with an ECL plus kit and autoradiography.

Cell growth assays

Cells were seeded in 6-well plates (40,000 cells/well) in DMEM with 10% FCS. Two days after seeding, cells were treated for 48 h and 72 h with the indicated concentration of chemicals or with the vehicle. For assay with CO (4 μg/ml/day), the treatment was repeated at 24 h and 48 h. At the indicated time, cells were trypsinized and counted using a Beckman-Coulter counter.

Cell invasion assays

Cells were seeded in 6-well plates (40,000 cells/well) in DMEM with 10% FCS. After 24 h, cells were pretreated for 24 h in the presence of the indicated chemicals or vehicle in DMEM with 2% FCS, then harvested and counted. CCK2R-WT or E151A cells (20,000 cells) and U87-MG cells (50,000 cells) were layered in serum free DMEM on the top of Nunc filters (8 mm diameter, 8 μm pore size) coated with growth factor-reduced Matrigel (250 μg/ml Matrigel®) in the presence of the appropriate chemical or vehicle. The bottom of the filter was filled with 10% FCS/DMEM. After 48 h at 37°C, cells that had invaded the Matrigel® and were attached to the lower face of the filter were fixed, stained with Giemsa stain, and counted under the microscope.

Statistical analysis

Statistical analysis was carried out using a Student's t-test for unpaired variables. *, **, and *** in the figure panels refer to probabilities (P) of <0.05, <0.01, and <0.001, respectively, compared with vehicle-treated cells. Change of reference is indicated in the legend of the figure when necessary.

RESULTS

To study the pattern of cholesterol esterification in cells expressing the CCK2R, we first used NIH-3T3 clones previously generated that express similar levels of wild-type (CCK2R-WT cells, clones WT4 and WT5) and mutated receptors (CCK2R-E151A cells, clones M1 and M40), as well as two clones expressing the empty vector (control cells, clones C20 and C50) (21). In the previous study, the constitutive activity of the CCK2R-E151A mutant expressed in NIH-3T3 cells was associated with enhanced cell proliferation and invasion as well as the formation of tumors in nude mice while no such effects were observed with cells expressing the CCK2R wild-type.

Cholesteryl ester formation is increased in tumor cells expressing the constitutively active mutant

As shown in **Fig. 1**, the pattern of basal cholesterol esterification in the CCK2R-E151A tumor cells (lanes 5 and 8) was compared with that of the nontumor CCK2R-WT (lanes 4 and 7) and control cells (lanes 3 and 6) by incubating cells over 24 h with ^{14}C-cholesterol. TLC analysis showed that the level of basal cholesterol esterification was similar in the two CCK2R-WT and control clones (16.7 ± 0.8 and 18.5 ± 0.9 pmol/10^{6}cell/24 h, respectively) while basal cholesterol esterification was 4 times greater in the CCK2R-E151A clones (69.5 ± 1.0 pmol/10^{6}cell/24 h), indicating that the increase in cholesteryl ester formation was the result of the constitutive activation of the mutant. Because similar results were obtained between the two wild-type clones and the two mutant clones, subsequent studies were realized with clones WT5 and M1. We then determined whether activation of the CCK2R-WT could induce cholesteryl ester formation by incubating the CCK2R-WT cells with ^{14}C-cholesterol and 10 nM gastrin. As shown in Fig. 1, lane 10, gastrin induced a 1.6-fold increase in cholesteryl ester formation (16.1 ± 0.3 pmol/10^6 cells/24 h) compared with cells treated with the vehicle (9.9 ± 0.2 pmol/10^6 cells/24 h) (Fig. 1, lane 9) when added every hour over 16 h to mimic sustained activation of the CCK2R and to overcome peptide degradation. These data indicate that the increase in cholesteryl ester formation measured in cells expressing the CCK2R-E151A mutant was not peculiar to the constitutively active mutant, because the CCK2R-WT showed cholesteryl ester formation when stimulated by gastrin.

The activity and expression of ACAT is increased in tumor cells expressing the constitutively active mutant

We next measured the mRNA level of the two isoforms of ACAT (ACAT1 and ACAT2) and carried out an enzymatic test to measure ACAT activity in cell lysates (35). As shown in **Fig. 2A**, the ACAT1 and ACAT2 mRNA levels were 1.69- and 1.39-fold higher in the CCK2R-E151A cells than in the CCK2R-WT cells. In addition, ACAT activity was 1.5-fold higher in tumor cells (133 ± 8 pmol/min/mg
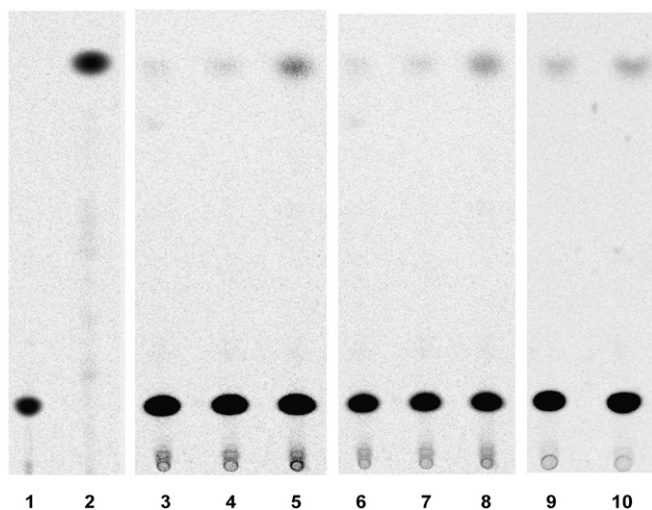
Fig. 1. Analysis of cholesterol esterification in CCK2R-E151A and CCK2R-WT cells. Analysis of cholesterol esterification in Mock, clone 50 and 20 (lane 3 and 6, respectively), CCK2R-WT cells, clone WT5 (lane 4) and clone WT2 (lane 7) or CCK2R-E151A cells, clone M40 (lane 5) or clone M1 (lane 8) incubated with ^{14}C-cholesterol (5.7 μM, 0.6 μCi) for 24 h and analysis of cholesterol esterification in CCK2R-WT cells incubated with ^{14}C-cholesterol (5.7 μM, 0.6 μCi) for 24 h and then treated with the solvent vehicle (lane 9) or with 10 nM gastrin (lane 10) added every hour for 16 h. After incubation, radiolabeled lipids were extracted, analyzed, and quantified as described in Materials and Methods. The positions of the different lipids have been determined using ^{14}C-labeled cholesterol (lane 1) and cholesteryl oleate (lane 2) as standards. Representative autoradiograms from three independent experiments are shown.

proteins) than in nontumor cells (86.7 ± 7.2 pmol/min/mg proteins) (Fig. 2B) and was completely inhibited by incubating cells with Sah 58-035 (5.3 ± 1.8 pmol/min/mg proteins), an inhibitor of the ACAT (36) (Fig. 2B). These data indicate that the formation of cholesteryl esters in the CCK2R-E151A cells is associated with an increase in the activity and expression of ACAT.

Erk activation and PKCζ are involved in cholesteryl ester formation in the CCK2R-E151A cells

To identify the intracellular signaling pathways coupling the constitutive CCK2R-E151A mutant to the formation of cholesteryl esters, we next examined the effects of different inhibitors of the main signaling pathways known to be activated by the CCK2R and/or the CCK2R-E151A mutant. As a control, we used the ACAT inhibitor Sah 58-035 (25 μM) that completely inhibited the formation of cholesteryl esters (**Fig. 3A**). As we showed that the CCK2R-E151A mutant constitutively activated ODC (21), we first studied the effect of polyamine depletion on cholesteryl ester formation by treating the CCK2R-E151A cells with α-difluoromethyl-ornithine (DFMO), a specific inhibitor of ODC (37). As shown in Fig. 3A, cholesteryl ester formation was not affected when cells were treated for 24 h with 5 mM DFMO, indicating that ODC activity is not involved in cholesteryl ester formation.

Because we had also shown that the CCK2R-E151A mutant was linked to an increase in phosphatidylinositol
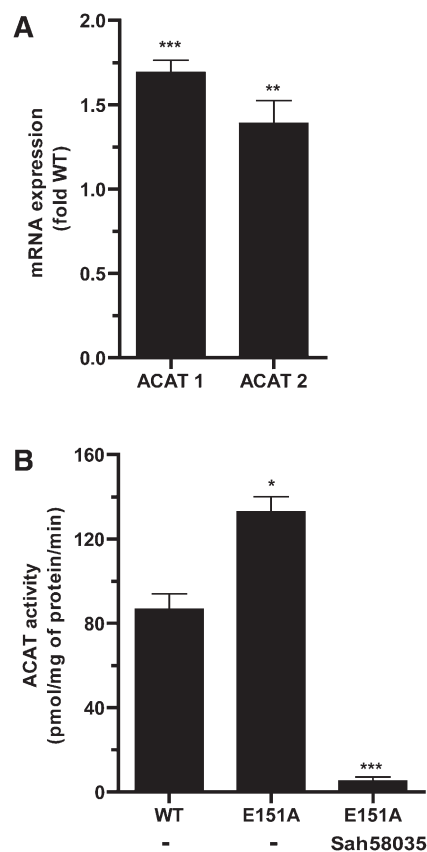

Fig. 2. Analysis of ACAT expression and activity in CCK2R-E151A and CCK2R-WT cells. A: ACAT1 and ACAT2 mRNA in the CCK2R-WT (WT) and CCK2R-E151A (E151A) cells was analyzed by real-time PCR. Expression of each gene was normalized to TBP with the values from the CCK2R-WT set as one. Values are the mean ± SEM of 4 separate experiments performed in triplicate. B: ACAT activity was assayed after treatment of cells for 24 h with the vehicle (WT and E151A) or with 25 μM Sah 58-035 (E151A + Sah58035) as described in Materials and Methods. Values are the mean ± SEM of three separate experiments.

hydrolysis (21), which is necessary to activate DAG-sensitive classical and novel PKCs (cPKC and nPKC, respectively), we tested inhibitors of these PKCs. As shown in Fig. 3A, the involvement of cPKC and nPKC was studied by using calphostin C, an inhibitor of diacylglycerol-dependent PKC isoforms and the calcium chelator BAPTA, an inhibitor of cPKC. These two inhibitors were ineffective in inhibiting cholesteryl ester formation; instead, the inhibition of these PKCs increased cholesteryl ester formation. By contrast, Gö6983, an inhibitor of cPKCs, nPKCs, and the atypical PKC, PKCζ (38), completely abolished the basal formation of cholesteryl esters (Fig. 3A). Because the involvement of cPKC and nPKC isoforms in the increase in cholesteryl ester formation was ruled out by experiments using calphostin C and BAPTA, these data would suggest an involvement of PKCζ. This was confirmed by using a dominant negative mutant of PKCζ that completely inhibited the constitutive formation of cholesteryl esters when expressed in the CCK2R-E151A cells (Fig. 3B). We then investigated the contribution of the mitogen-activated protein kinases MAP-kinase kinase (MEK)/ERK1/2, a pathway activated
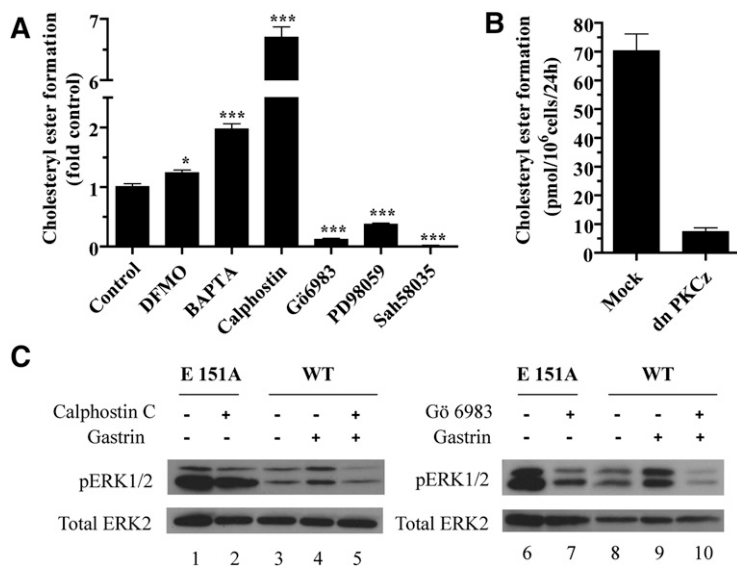
Fig. 3. Inhibition of cholesterol esterification in CCK2R-E151A cells. Cholesterol ester formation was determined as described in Materials and Methods. A: After 24 h treatment of the CCK2R-E151A cells with ^{14}C-cholesterol (5.7 µM, 0.6 µCi) and with either the solvent vehicle (control) or the indicated inhibitors: Sah 58-035 (25 µM), DFMO (5 mM), BAPTA (10 µM), Calphostin C (1 µM), Gö6983 (10 µM), and PD 98053 (50 µM). Values are expressed relative to that of cells treated with the solvent vehicle (control) and are the mean ± SEM of three separate experiments performed in triplicate. B: After 24 h treatment of the CCK2R-E151A cells, transfected with the empty vector (Mock) or the dominant negative PKCζ (dnPKCz) plasmids, with ^{14}C-cholesterol (5.7 µM, 0.6 µCi). Values are the mean ± SEM of 3 separate experiments performed in triplicate. C: CCK2R-E151A and CCK2R-WT cells were incubated overnight either with the solvent vehicle, calphostin C (1 µM) or Gö6983 (10 µM), then stimulated or not with 10 nM gastrin for 10 min. Phosphorylated (p-ERK1/2) or total (ERK2) expression levels were analyzed as described in Materials and Methods. Representative blots from three independent experiments are shown.

by the CCK2R that has been reported to be dependent on both DAG-sensitive and DAG-insensitive PKC. As shown in Fig. 3A, the formation of cholesteryl esters was prevented by incubating the CCK2R-E151A cells with the MEK inhibitor PD98059. To determine whether ERK1/2 is a downstream target of PKCζ, the CCK2R-E151A cells were incubated with calphostin C or Gö6983 and ERK1/2 phosphorylation was analyzed by western blotting. As a comparison, similar treatments were carried out with CCK2R-WT cells before activation with gastrin. As shown in Fig. 3C, an increased basal phosphorylation of ERK1/2 was observed in CCK2R-E151A cells compared with CCK2R-WT cells (lane 1 vs. 3 or lane 6 vs. 8), showing that ERK1/2 were constitutively activated in the CCK2R-E151A cells. Treatment with calphostin C did not inhibit ERK1/2 phosphorylation in the CCK2R-E151A cells (lane 2 vs. 1) but reduced gastrin-stimulated ERK1/2 phosphorylation in the CCK2R-WT cells (lane 5 vs. 4). Treatment with Gö6983 inhibited ERK1/2 phosphorylation in the CCK2R-E151A cells (lane 7 vs. 6) and gastrin-stimulated ERK1/2 phosphorylation in the CCK2R-WT cells (lane 10 vs. 9). Taken together, these results indicated that the ERK1/2 in the CCK2R-E151A cells is a downstream target of PKCζ.

Inhibition of cholesteryl ester formation has an impact on CCK2R-E151A cell proliferation and invasion

We then investigated the role of the cholesteryl ester formation to the enhanced proliferation and invasion previously reported for the CCK2R-E151A cells (21). Treatment for 48 h and 72 h with the ACAT inhibitor Sah 58-035 at 25 µM, a concentration that completely inhibited cholesterol esterification, reduced in a time-dependent manner the growth rate of the CCK2R-E151A cells. A decrease of 34%, from 1.8- to 1.2-fold, was measured at 72 h compared with the CCK2R-WT cells (**Fig. 4A**). Similarly, treatment with 25 µM Sah 58-035 for 48 h reduced by 73% the invasiveness of the CCK2R-E151A cells (Fig. 4B).

Cholesteryl esters added to the culture medium stimulate the proliferation and invasiveness of the nontumor CCK2R-WT cells

To further investigate the impact of cholesteryl esters on cell proliferation and invasion, we studied the effects of cholesteryl esters added to the culture medium of the CCK2R-WT cells. For this purpose, the CCK2R-WT cells were cultured in medium containing 4 µg/ml cholesteryl
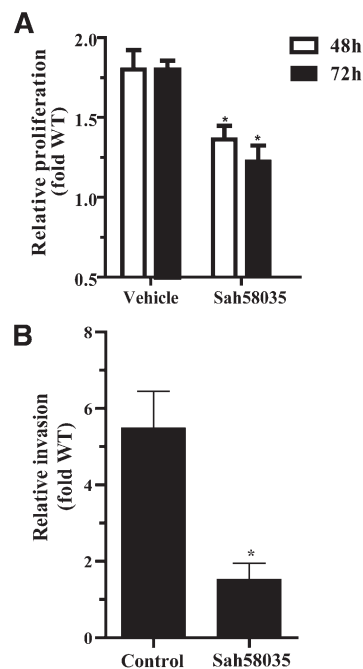

Fig. 4. Effects of ACAT inhibitor on CCK2R-E151A cell proliferation and invasion. A: After 48 h and 72 h treatment with the solvent vehicle (control) or 25 µM Sah 58-035, proliferation of the CCK2R-E151A cells was assayed by cell counting. B: After 24 h treatment with the solvent vehicle or 25 µM Sah 58-035, cell invasion was assayed using matrigel-coated filters as described in Materials and Methods. After 48 h, cells on the lower surface of the filters were stained and counted under a phase-contrast microscope. Values are expressed relative to that of the CCK2R-WT cells and are the mean ± SEM of 3 separate experiments.

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oleate (CO), the main cholesteryl ester formed intracellularly (39) and compared with CCK2R-WT and CCK2R-E151A cell culture in the absence of CO. As shown in **Fig. 5A**, during the first 48 h of treatment, not significant changes were observed. By contrast, after 72 h of treatment, the growth rate of the CCK2R-WT cells cultured with CO was significantly increased and close to that of the CCK2R-E151A cells (1.6-fold vs. 1.8-fold, respectively). In addition, the CCK2R-WT cells cultured with CO for 48 h displayed a 3.5-fold greater invasive capacity than vehicle-treated CCK2R-WT cells. For comparison, the CCK2R-E151A cells had a 5.5-fold higher invasive capacity (Fig. 5B).

Inhibition of cholesteryl ester formed through the activation of the CCK2R reduced U87 cell proliferation and invasion

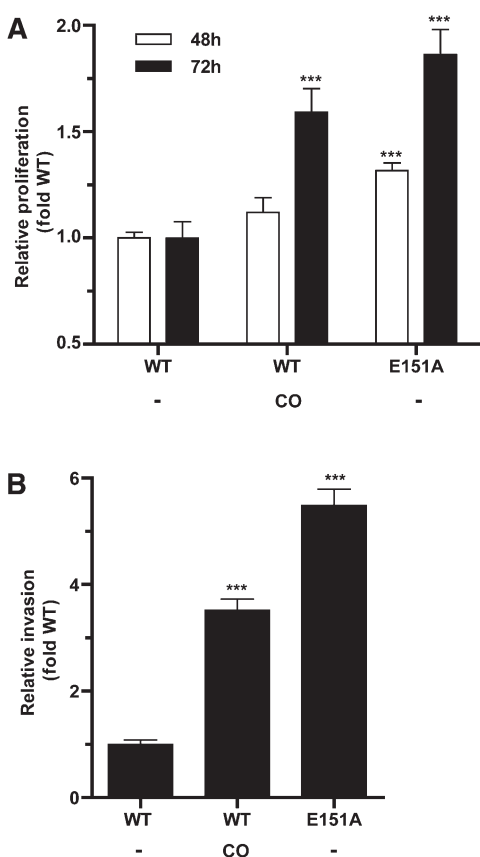To further characterize the importance of cholesteryl esters in proliferation and invasion of cells expressing an

Fig. 5. Effect of cholesteryl oleate on CCK2R-WT cell proliferation and invasion. A: CCK2R-WT cells (WT) or CCK2R-E151A cells (E151A) were treated with 4 μg/ml cholesteryl oleate (+ CO) or the solvent vehicle (− CO) for 48 h or 72 h and proliferation was assayed by cell counting. B: Invasion by CCK2R-WT cells (WT) pretreated for 24 h with cholesteryl oleate (4 μg/ml) and layered on the top of Matrigel-coated filters in serum-free medium containing cholesteryl oleate (4 μg/ml) for 48 h (+ CO) and invasion by CCK2R-WT cells (WT) and CCK2R-E151A cells (E151A) treated similarly with the solvent vehicle (− CO). After 48 h, cells on the lower surface of the filters were stained and counted. Values are expressed relative to that of the CCK2R-WT cells treated with the solvent vehicle (−CO) and are the mean ± SEM of three separate experiments.

activated CCK2R, we evaluated cholesteryl ester production in U87 human glioma cells, a tumor cell line in which an autocrine activation of the CCK2R by its agonist has been shown to control cell growth (20). As shown in **Fig. 6A**, the pattern of cholesteryl ester formation was assessed by incubating U87 cells over 24 h with ^{14}C-cholesterol in the absence (lane 3) or presence of Sah 58-035 (lane 4). Sah 58-035 inhibited by 90% cholesterol esterification $(86.5 \pm 1.9$ vs. 7.7 ± 0.9 pmol/10^6 cell/24 h; Fig. 6B). To characterize whether the activated CCK2R could mediate cholesteryl ester formation, we performed similar experiments in the presence of L365260 and YM022 (lanes 9 and 10), two selective and potent antagonists of the CCK2R (40, 41). L365260 and YM022 decreased by 93% and 65%, respectively, the esterification of cholesterol $(6.5 \pm 1.1$ and 30.0 ± 3.7 pmol/10^6cell/24 h; Fig. 6B). In a similar manner, ACAT activity was completely inhibited with Sah 58-035 and by 84% and 73% with L365 260 and YM22, respectively (Fig. 6C).

We then characterized whether PKCζ and ERK1/2 were also involved in cholesteryl ester formation in U87 cells. As shown in Fig. 6A, cholesteryl ester production was not inhibited by BAPTA (lane 5) or calphostin C (lane 6) but instead both inhibitors increased their formation. In contrast, cholesterol esterification was inhibited by 93% and 85% with Gö6983 (lane 7) and PD98059 (lane 8), respectively, indicating that cholesteryl ester formation is mainly dependent on PKC ζ and MEK/ERK1/2. We then determined whether cholesteryl esters formed by the activated CCK2R were involved in U87 proliferation and invasion. The growth rate of U87 cells was shown inhibited in a time-dependent manner by Sah 58-035, L365260, and YM022 (Fig. 6D). After 72 h treatment, U87 proliferation was inhibited by 32% with Sah 58-035 and by 38% and 56% with L365260 and YM022, respectively (Fig. 6D). In addition, the invasiveness of the U87 cells was inhibited by 60% with Sah 58-035, 80% with L365206, and 61% with YM22 (Fig. 6E). Together, these results indicate that cholesteryl ester formation produced by the CCK2R contributes to the proliferation and invasiveness of these cells.

DISCUSSION

Different studies suggest that cholesteryl esters and ACAT are involved in various aspects of tumor transformation. Indeed, in cells isolated from patients with lymphoblastic T-cell leukemia, a positive correlation between increased cholesterol esterification and growth rate was associated with higher ACAT mRNA levels, suggesting a link between cholesteryl ester formation and cell proliferation (30, 42). In addition, higher expression and activity of ACAT1 protein in clear cell type renal carcinoma compared with normal kidney tissues have been shown associated with increased levels of cholesteryl ester and with tumor grade (32). In line of these results, we were able to demonstrate in the present study the importance of cholesteryl esters and ACAT in the proliferation and invasiveness of two tumor cell lines through the sustained activation of the CCK2R.
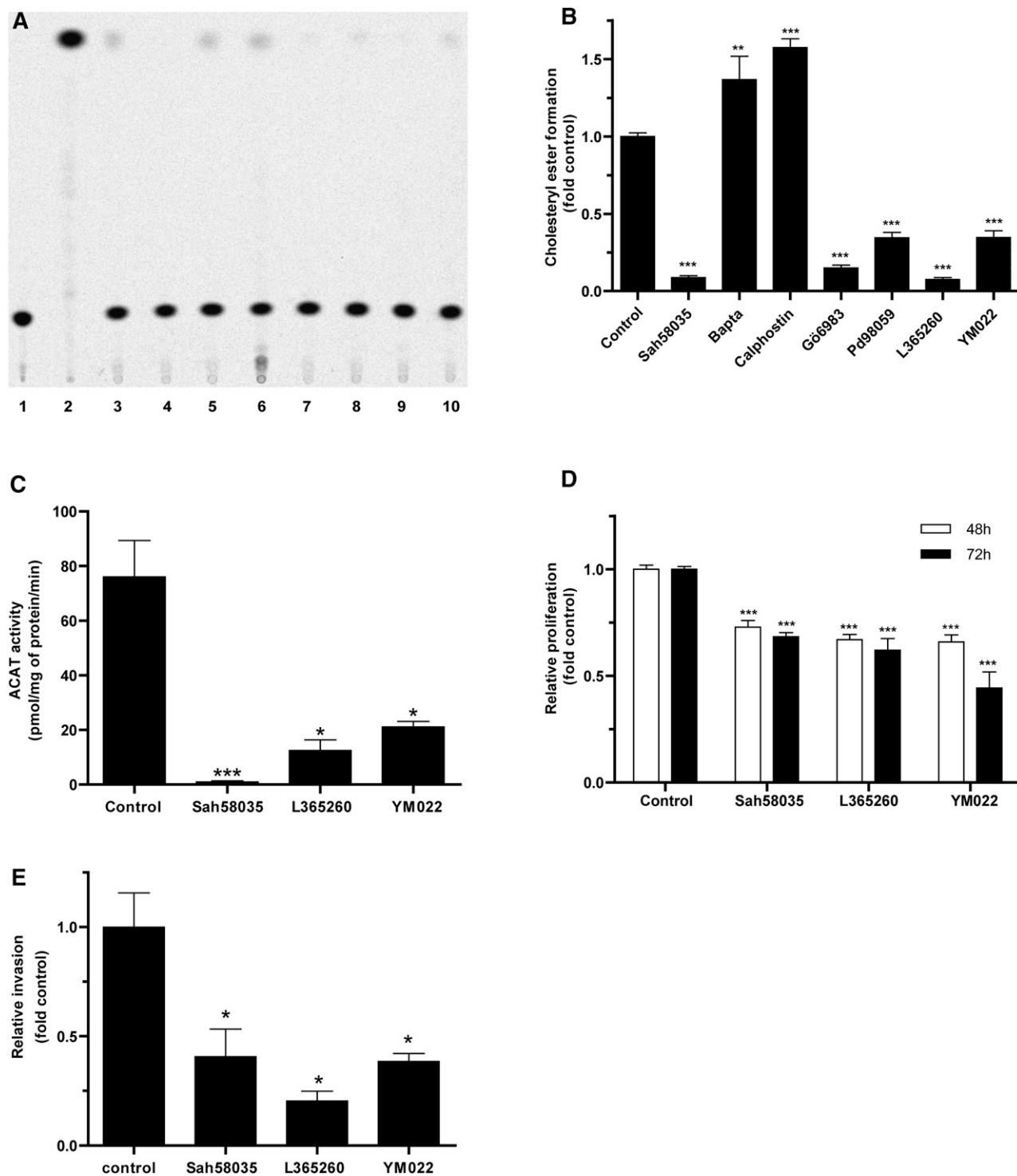
Fig. 6. Inhibition of cholesterol esterification in U87 cells and effect on cell proliferation and invasion. A: Analysis of cholesterol esterification in U87 cells treated for 24 h with the solvent vehicle (control, lane 3), 25 µM Sah 58-035 (lane 4), 10 µM BAPTA AM (lane 5), 1 µM calphostin C (lane 6), 10 µM Gö6983 (lane 7), 50 µM PD98059 (lane 8), 0.1 µM L365,260 (lane 9), and 0.1 µM YM022 (lane 10) in the presence of ^{14}C-cholesterol (5.7 µM, 0.6 µCi). Radiolabeled lipids were extracted, analyzed, and quantified as described in Materials and Methods. The positions of the different lipids have been determined using ^{14}C-labeled cholesterol (lane 1) and ^{14}C-labeled cholesteryl oleate (lane 2) as standards. A representative autoradiogram from three independent experiments is shown. B: Quantitative analysis of cholesterol ester formation in U87 cells treated with the different chemicals used in A or with the solvent vehicle (control). C: ACAT activity was assayed as described in Materials and Methods after treatment of cells for 24 h with the solvent vehicle (control), 25 µM Sah 58-035, 0.1 µM L365 260, or 0.1 µM YM022. Values are the mean ± SEM of three separate experiments. D: U87 cells were treated for the indicated time with the solvent vehicle (control), 25 µM Sah 58-035, 0.1 µM L365 260, and 0.1 µM YM022 and proliferation was assayed by cell counting. E: After 24 h pretreatment with either the solvent vehicle, 25 µM Sah 58-035, 0.1 µM L365 260, or 0.1 µM YM022, cell invasion was assayed using matrigel-coated filters as described in Materials and Methods. After 48 h, cells on the lower surface of the filters were stained and counted under a phase-contrast microscope. B–E: Values are expressed relative to that of cells treated with the solvent vehicle (control) and are the mean ± SEM of three to six separate experiments performed in triplicate.

We first provide evidence for a significant increase in cholesterol esterification in tumor cells expressing a constitutively active CCK2R mutant associated with an enhanced proliferation and invasion of these cells. Indeed, inhibition of cholesteryl ester formation by inhibiting ACAT reduced by 34% the growth of these cells and by 75% their invasiveness. The increase in cholesterol esterification in these tumor cells was associated with an upregulation of ACAT1 and ACAT2 expression and an increase in ACAT activity as reported for leukemia and renal carcinoma (30, 32, 42). The importance of cholesteryl esters in cell proliferation and invasion mediated by an activated CCK2R was also demonstrated in human U87 glioma cells, an autocrine model of activation of the CCK2R (20). Inhibition of cholesteryl ester formation either by blocking ACAT or the CCK2R with two selective CCK2R antagonists reduced significantly and in a similar way the growth and invasiveness of these cells. These results validated the presence of an endogenous autocrine mechanism of receptor activation that induced cholesteryl ester formation.

It has to be noted that, in both tumor cells, the inhibition of cholesterol esterification by the ACAT inhibitor and CCK2R antagonists strongly impacts cell invasion. In line with these studies, increased levels of cholesteryl esters in glioma tissues and in surrounding infiltrated areas of human brain have been reported compared with normal material while cholesterol was significantly lower in tumor tissue (43), suggesting a possible involvement of cholesteryl esters in the development and progression of these tumors that remain untreatable. In addition, important amounts of cholesteryl esters were detected only in tumor lesions at the highest grade of malignancy in cerebral, renal, and prostatic neoplastic tissues, further supporting the concept that they may participate in the aggressivity and progression of different tumors (31, 44–47). In accordance with these different studies, the role of cholesteryl esters in cell proliferation and invasion was further demonstrated by culturing the nontumor cells expressing the wild-type CCK2R in the presence of cholesteryl oleate. In these conditions, the growth rate and the invasiveness of nontumor cells reached that of the tumor cells.

In both tumor models, stimulation of cholesterol esterification was characterized as being mainly dependent on atypical PKCζ and MEK/ERK1/2, a signaling pathway involved in mitogenic processes. However, we observed an increase in cholesteryl ester formation when DAG- and calcium-sensitive PKC(s) were inhibited by calphostin C and BAPTA, suggesting that these kinases are activated in both cells and exert a repressive effect on cholesteryl ester formation. The activation of DAG- and calcium-sensitive PKC(s) in these cells is in agreement with the constitutive activation of phospholipase C reported for the CCK2R mutant in the CCK2R-E151A cells (21) and with the autocrine activation of the CCK2R in U87 cells that was shown coupled to inositol phosphate production (20). Thus, cholesterol ester formation seems regulated by the balance between the opposing effects of DAG-sensitive and DAG-insensitive PKC/ERK1/2 activation. Similar results, activation

by ERK1/2 and repression by DAG-sensitive PKC, have been reported to regulate the rate of cholesterol esterification in human monocyte-derived macrophages (48).

How cholesterol esterification by ACAT and cholesteryl esters influences tumor cell proliferation and invasion remains an open question. It was reported that inhibition of ACAT enhances the pool of free cholesterol available for conversion into oxysterols (49). Oxysterols have been considered as potential chemotherapeutic agents for the control of cellular growth of both normal and cancer cells (27). In line with these results, we have recently characterized the mechanism of action of anti-cancer drugs such as Tamoxifen targeting the anti-estrogen binding site (50, 51). We showed that Tamoxifen and selective anti-estrogen binding site ligands induced tumor cell differentiation and apoptosis through the production of oxysterols and that this mechanism could account for their anti-tumor and chemopreventative actions. Interestingly, we previously reported that Tamoxifen is an inhibitor of ACAT (35). This effect could also favor the production of oxysterols that are necessary for its anti-tumor effects (50, 51). Based on these different studies, it could be hypothesized that increased cholesterol esterification could be a mechanism used by tumor cells to quench or prevent the cytotoxic effects of oxysterols. These different studies and the present study suggest that ACAT is an important target for anti-cancer therapy or prevention.

In conclusion, the originality of our study is to link the activation of a G protein-coupled receptor to cell proliferation and invasion via the stimulation of the ACAT/cholesterol esterification pathway. The ACAT/cholesterol esterification pathway thus appears as a new signaling pathway for the CCK2R that should be targeted for anti-cancer drug screening and discovery.

REFERENCES

1. Rehfeld, J. F. 2004. Cholecystokinin. *Best Pract. Res. Clin. Endocrinol. Metab.* **18:** 569–586.
2. Dockray, G., R. Dimaline, and A. Varro. 2005. Gastrin: old hormone, new functions. *Pflugers Arch.* **449:** 344–355.
3. Iwase, K., B. M. Evers, M. R. Hellmich, Y. S. Guo, S. Higashide, H. J. Kim, and C. M. Townsend, Jr. 1997. Regulation of growth of human gastric cancer by gastrin and glycine-extended progastrin. *Gastroenterology.* **113:** 782–790.
4. Caplin, M., K. Khan, S. Grimes, D. Michaeli, K. Savage, R. Pounder, and A. Dhillon. 2001. Effect of gastrin and anti-gastrin antibodies on proliferation of hepatocyte cell lines. *Dig. Dis. Sci.* **46:** 1356–1366.
5. Moody, T. W., and R. T. Jensen. 2001. CI-988 inhibits growth of small cell lung cancer cells. *J. Pharmacol. Exp. Ther.* **299:** 1154–1160.
6. Rozengurt, E., and J. H. Walsh. 2001. Gastrin, CCK, signaling, and cancer. *Annu. Rev. Physiol.* **63:** 49–76.
7. Stubbs, M., K. Khan, R. G. Wickremasinghe, K. Ganeshaguru, and M. E. Caplin. 2005. CCK2 gastrin receptor as a potential target for therapy in leukaemia cell lines. *Oncol. Rep.* **14:** 1055–1058.
8. Savage, K., H. A. Waller, M. Stubbs, K. Khan, S. A. Watson, P. A. Clarke, S. Grimes, D. Michaeli, A. P. Dhillon, and M. E. Caplin. 2006. Targeting of cholecystokinin B/gastrin receptor in colonic, pancreatic and hepatocellular carcinoma cell lines. *Int. J. Oncol.* **29:** 1429–1435.

9. Staley, J., R. T. Jensen, and T. W. Moody. 1990. CCK antagonists interact with CCK-B receptors on human small cell lung cancer cells. *Peptides.* **11:** 1033–1036.

10. Nagata, A., M. Ito, N. Iwata, J. Kuno, H. Takano, O. Minowa, K. Chihara, T. Matsui, and T. Noda. 1996. G protein-coupled cholecystokinin-B/gastrin receptors are responsible for physiological cell growth of the stomach mucosa in vivo. *Proc. Natl. Acad. Sci. USA.* **93:** 11825–11830.

11. Schmitz, F., M. N. Goke, J. M. Otte, H. Schrader, B. Reimann, M. L. Kruse, E. G. Siegel, J. Peters, K. H. Herzig, U. R. Folsch, et al. 2001. Cellular expression of CCK-A and CCK-B/gastrin receptors in human gastric mucosa. *Regul. Pept.* **102:** 101–110.

12. Smith, J. P., A. H. Shih, M. G. Wotring, P. J. McLaughlin, and I. S. Zagon. 1998. Characterization of CCK-B/gastrin-like receptors in human gastric carcinoma. *Int. J. Oncol.* **12:** 411–419.

13. Reubi, J. C., J. C. Schaer, and B. Waser. 1997. Cholecystokinin (CCK)-A and CCK-B/gastrin receptors in human tumors. *Cancer Res.* **57:** 1377–1386.

14. McWilliams, D. F., S. A. Watson, D. M. Crosbee, D. Michaeli, and R. Seth. 1998. Coexpression of gastrin and gastrin receptors (CCK-B and delta CCK-B) in gastrointestinal tumour cell lines. *Gut.* **42:** 795–798.

15. Caplin, M., K. Khan, K. Savage, J. Rode, A. Varro, D. Michaeli, S. Grimes, B. Brett, R. Pounder, and A. Dhillon. 1999. Expression and processing of gastrin in hepatocellular carcinoma, fibrolamellar carcinoma and cholangiocarcinoma. *J. Hepatol.* **30:** 519–526.

16. Blackmore, M., and B. H. Hirst. 1992. Autocrine stimulation of growth of AR4–2J rat pancreatic tumour cells by gastrin. *Br. J. Cancer.* **66:** 32–38.

17. Caplin, M., K. Savage, K. Khan, B. Brett, J. Rode, A. Varro, and A. Dhillon. 2000. Expression and processing of gastrin in pancreatic adenocarcinoma. *Br. J. Surg.* **87:** 1035–1040.

18. Smith, J. P., A. Shih, Y. Wu, P. J. McLaughlin, and I. S. Zagon. 1996. Gastrin regulates growth of human pancreatic cancer in a tonic and autocrine fashion. *Am. J. Physiol.* **270:** R1078–R1084.

19. Grabowska, A. M., and S. A. Watson. 2007. Role of gastrin peptides in carcinogenesis. *Cancer Lett.* **257:** 1–15.

20. Oikonomou, E., M. Buchfelder, and E. F. Adams. 2008. Cholecystokinin (CCK) and CCK receptor expression by human gliomas: evidence for an autocrine/paracrine stimulatory loop. *Neuropeptides.* **42:** 255–265.

21. Gales, C., D. Sanchez, M. Poirot, S. Pyronnet, L. Buscail, D. Cussac, L. Pradayrol, D. Fourmy, and S. Silvente-Poirot. 2003. High tumorigenic potential of a constitutively active mutant of the cholecystokinin 2 receptor. *Oncogene.* **22:** 6081–6089.

22. Chang, A. J., D. H. Song, and M. M. Wolfe. 2006. Attenuation of peroxisome proliferator-activated receptor gamma (PPARgamma) mediates gastrin-stimulated colorectal cancer cell proliferation. *J. Biol. Chem.* **281:** 14700–14710.

23. Hansen, T. O., R. Borup, T. Marstrand, J. F. Rehfeld, and F. C. Nielsen. 2008. Cholecystokinin-2 receptor mediated gene expression in neuronal PC12 cells. *J. Neurochem.* **104:** 1450–1465.

24. Simons, K., and E. Ikonen. 2000. How cells handle cholesterol. *Science.* **290:** 1721–1726.

25. Pucadyil, T. J., and A. Chattopadhyay. 2006. Role of cholesterol in the function and organization of G-protein coupled receptors. *Prog. Lipid Res.* **45:** 295–333.

26. Ikonen, E. 2008. Cellular cholesterol trafficking and compartmentalization. *Nat. Rev. Mol. Cell Biol.* **9:** 125–138.

27. Schroepfer, G. J., Jr. 2000. Oxysterols: modulators of cholesterol metabolism and other processes. *Physiol. Rev.* **80:** 361–554.

28. Gebhard, R. L., R. V. Clayman, W. F. Prigge, R. Figenshau, N. A. Staley, C. Reesey, and A. Bear. 1987. Abnormal cholesterol metabolism in renal clear cell carcinoma. *J. Lipid Res.* **28:** 1177–1184.

29. Dessi, S., B. Batetta, C. Anchisi, P. Pani, P. Costelli, L. Tessitore, and F. M. Baccino. 1992. Cholesterol metabolism during the growth of a rat ascites hepatoma (Yoshida AH-130). *Br. J. Cancer.* **66:** 787–793.

30. Dessi, S., B. Batetta, A. Pani, O. Spano, F. Sanna, M. Putzolu, R. Bonatesta, S. Piras, and P. Pani. 1997. Role of cholesterol synthesis and esterification in the growth of CEM and MOLT4 lymphoblastic cells. *Biochem. J.* **321:** 603–608.

31. Tosi, M. R., G. Bottura, P. Lucchi, A. Reggiani, A. Trinchero, and V. Tugnoli. 2003. Cholesteryl esters in human malignant neoplasms. *Int. J. Mol. Med.* **11:** 95–98.

32. Matsumoto, K., Y. Fujiwara, R. Nagai, M. Yoshida, and S. Ueda. 2008. Expression of two isozymes of acyl-coenzyme A: cholesterol acyltransferase-1 and -2 in clear cell type renal cell carcinoma. *Int. J. Urol.* **15:** 166–170.

33. Bligh, E. G., and W. J. Dyer. 1959. A rapid method of total lipid extraction and purification. *Can. J. Biochem. Physiol.* **37:** 911–917.

34. Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72:** 248–254.

35. de Medina, P., B. L. Payre, J. Bernad, I. Bosser, B. Pipy, S. Silvente-Poirot, G. Favre, J. C. Faye, and M. Poirot. 2004. Tamoxifen is a potent inhibitor of cholesterol esterification and prevents the formation of foam cells. *J. Pharmacol. Exp. Ther.* **308:** 1165–1173.

36. Ross, A. C., K. J. Go, J. G. Heider, and G. H. Rothblat. 1984. Selective inhibition of acyl coenzyme A:cholesterol acyltransferase by compound 58–035. *J. Biol. Chem.* **259:** 815–819.

37. Poulin, R., L. Lu, B. Ackermann, P. Bey, and A. E. Pegg. 1992. Mechanism of the irreversible inactivation of mouse ornithine decarboxylase by alpha-difluoromethylornithine. Characterization of sequences at the inhibitor and coenzyme binding sites. *J. Biol. Chem.* **267:** 150–158.

38. Gschwendt, M., S. Dieterich, J. Rennecke, W. Kittstein, H. J. Mueller, and F. J. Johannes. 1996. Inhibition of protein kinase C mu by various inhibitors. Differentiation from protein kinase c isoenzymes. *FEBS Lett.* **392:** 77–80.

39. Brown, M. S., Y. K. Ho, and J. L. Goldstein. 1980. The cholesteryl ester cycle in macrophage foam cells. Continual hydrolysis and re-esterification of cytoplasmic cholesteryl esters. *J. Biol. Chem.* **255:** 9344–9352.

40. Nishida, A., K. Miyata, R. Tsutsumi, H. Yuki, S. Akuzawa, A. Kobayashi, T. Kamato, H. Ito, M. Yamano, Y. Katuyama, et al. 1994. Pharmacological profile of (R)-1-[2,3-dihydro-1-(2'-methylphenacyl)-2-oxo- 5-phenyl-1H–1,4-benzodiazepin-3-yl]-3-(3-methylphenyl)urea (YM022), a new potent and selective gastrin/cholecystokinin-B receptor antagonist, in vitro and in vivo. *J. Pharmacol. Exp. Ther.* **269:** 725–731.

41. Chang, R. S., T. B. Chen, M. G. Bock, R. M. Freidinger, R. Chen, A. Rosegay, and V. J. Lotti. 1989. Characterization of the binding of [3H]L-365,260: a new potent and selective brain cholecystokinin (CCK-B) and gastrin receptor antagonist radioligand. *Mol. Pharmacol.* **35:** 803–808.

42. Batetta, B., A. Pani, M. Putzolu, F. Sanna, R. Bonatesta, S. Piras, O. Spano, M. F. Mulas, and S. Dessi. 1999. Correlation between cholesterol esterification, MDR1 gene expression and rate of cell proliferation in CEM and MOLT4 cell lines. *Cell Prolif.* **32:** 49–61.

43. Nygren, C., H. von Holst, J. E. Mansson, and P. Fredman. 1997. Increased levels of cholesterol esters in glioma tissue and surrounding areas of human brain. *Br. J. Neurosurg.* **11:** 216–220.

44. Beckonert, O., J. Monnerjahn, U. Bonk, and D. Leibfritz. 2003. Visualizing metabolic changes in breast-cancer tissue using 1H-NMR spectroscopy and self-organizing maps. *NMR Biomed.* **16:** 1–11.

45. Tosi, M. R., and V. Tugnoli. 2005. Cholesteryl esters in malignancy. *Clin. Chim. Acta.* **359:** 27–45.

46. Tugnoli, V., M. R. Tosi, A. Tinti, A. Trinchero, G. Bottura, and G. Fini. 2001. Characterization of lipids from human brain tissues by multinuclear magnetic resonance spectroscopy. *Biopolymers.* **62:** 297–306.

47. Tugnoli, V., A. Reggiani, R. Beghelli, V. Tomaselli, A. Trinchero, and M. R. Tosi. 2003. Magnetic resonance spectroscopy and high performance liquid chromatography of neoplastic human renal tissues. *Anticancer Res.* **23:** 1541–1548.

48. Napolitano, M., M. Avella, N. T. Goode, K. M. Botham, and E. Bravo. 2004. Cholesterol esterification in human monocyte-derived macrophages is inhibited by protein kinase C with dual roles for mitogen activated protein kinases. *Cell Biol. Int.* **28:** 717–725.

49. Post, S. M., J. P. Zoeteweij, M. H. Bos, E. C. de Wit, R. Havinga, F. Kuipers, and H. M. Princen. 1999. Acyl-coenzyme A:cholesterol acyltransferase inhibitor, avasimibe, stimulates bile acid synthesis and cholesterol 7alpha-hydroxylase in cultured rat hepatocytes and in vivo in the rat. *Hepatology.* **30:** 491–500.

50. Payre, B., P. de Medina, N. Boubekeur, L. Mhamdi, J. Bertrand-Michel, F. Terce, I. Fourquaux, D. Goudouneche, M. Record, M. Poirot, et al. 2008. Microsomal antiestrogen-binding site ligands induce growth control and differentiation of human breast cancer cells through the modulation of cholesterol metabolism. *Mol. Cancer Ther.* **7:** 3707–3718.

51. de Medina, P., B. Payre, N. Boubekeur, J. Bertrand-Michel, F. Terce, S. Silvente-Poirot, and M. Poirot. 2009. Ligands of the antiestrogen binding site induce active cell death and autophagy in human breast cancer cells through the modulation of cholesterol metabolism. *Cell Death Differ.* **16:** 1372–1384.