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PI3K/AKT, JNK, and ERK Pathways Are Not Crucial for the Induction of Cholesterol Biosynthesis Gene Transcription in Intestinal Epithelial Cells Following Treatment with the Potato Glycoalkaloid α-Chaconine

TAFADZWA MANDIMIKA,[†,‡] HAKAN BAYKUS,[†] JENNEKE POORTMAN,[†] CUTBERTO GARZA,[§] HARRY KUIPER,[†] AND AD PEIJNENBURG*,[†]

RIKILT—Institute of Food Safety, Wageningen University and Research Centre, P.O. Box 230, 6700 AE Wageningen, The Netherlands, Division of Nutritional Sciences, Cornell University, Ithaca, New York 14853, and Office of the Provost, Boston College, Chestnut Hill, Massachusetts 02467

We previously reported that exposure of the intestinal epithelial Caco-2 cell line to noncytotoxic concentrations of potato glycoalkaloids resulted in increased expression of cholesterol biosynthesis genes. Genes involved in mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3-kinase (PI3K)/v-akt murine thymoma viral oncogene homologue (AKT) pathways and their downstream effectors such as Jun, c-Myc, and Fos also were induced. MAPK and PI3K/AKT pathways have been described to regulate the activity of sterol regulatory element binding transcription factors (SREBPs) and consequently the expression of cholesterol biosynthesis genes. In this study, to understand the mechanism of induction of cholesterol biosynthesis upon α-chaconine treatment, its effect on SREBP-2 protein levels was investigated. We also examined whether MAPK and PI3K/AKT pathways are required for the observed induction of these genes following exposure of cells to α-chaconine. Differentiated Caco-2 cells were pretreated with LY294002 (PI3K inhibitor), PD98059 (MEK1 inhibitor), or SP600125 (JNK inhibitor) or a combination of all inhibitors for 24 h prior to coincubation with 10 μM α-chaconine for 6 h. Significant increases in precursor and mature protein levels of SREBP-2 were observed after α-chaconine exposure. We also observed that α-chaconine treatment resulted in significant phosphorylation of AKT, extracellular signal related protein kinase (ERK), and c-jun N terminal protein kinase (JNK) but not that of p38. In general, the kinase inhibitor experiments revealed that phosphorylation of kinases of PI3K/AKT, ERK, and JNK pathways was not crucial for the induction of expression of cholesterol biosynthesis genes, with the exception of SC5DL. The transcription of this later gene was reduced when all three pathways were inhibited. On the basis of these results, it can be postulated that other mechanisms, which may be independent of the MAPK and PI3K/AKT pathways, including possibly post-translational activation of SREBP-2, may be more pivotal for the induction of cholesterol biosynthesis genes following exposure of intestinal cells to α-chaconine.

KEYWORDS: Glycoalkaloids; α-chaconine; MAPK; PI3K/AKT; cholesterol biosynthesis

INTRODUCTION

Potatoes (*Solanum tuberosum* L.) contain the toxins α-chaconine and α-solanine. These account for 95% of potatoes' total glycoalkaloids (*1*). Glycoalkaloids at high levels (3−6 mg/kg body weight) may have toxic effects on human health (*2*) including gastrointestinal disturbances, increased heartbeat, hemolysis, and neurotoxic effects (*3*). Reported toxicities are due mainly to acetylcholinesterase inhibition and cell membrane disruption that affect digestive and other organs (*4*). Toxicities induced in other species include hepatoxicity in mice (*5*), increased hepatic ornithine decarboxylase activity in rats (*6*), craniofacial malformations in hamsters (*7*), and anatomical developmental toxicities in frog embryos (*8*).

The most well-documented mechanism of glycoalkaloid toxicity is the disruption of membrane integrity, which is caused by the formation of destabilizing complexes between the lipophilic moiety of glycoalkaloids and the cholesterol present in membranes (*9, 10*). At certain sterol threshold concentrations, glycoalkaloids can form irreversibly glycoalkaloid/sterol com-

* To whom correspondence should be addressed. Tel: + 31-317-475462. Fax: + 31-317-417717. E-mail: ad.peijnenburg@wur.nl.
[†] Wageningen University and Research Centre.
[‡] Cornell University.
[§] Boston College.

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plexes in cell membranes resulting in rapid loss of membrane barrier integrity (*9, 10*). Previously, we reported on cytotoxicity and gene expression studies in which Caco-2 cells were treated with potato glycoalkaloids, particularly α-chaconine (*11, 12*). The Caco-2 cell line was chosen as it is widely used as an in vitro model system for the intestinal epithelium (*13*), which is one of the main targets of glycoalkaloid activity. In these latter studies, we observed increased lactate dehydrogenase leakage in cells following exposure to increasing concentrations of glycoalkaloids, an indicator of increased membrane disruption. In addition, we observed elevated levels of cholesterol biosynthesis gene expression in cells treated with α-chaconine, prior to changes reflective of cytotoxicity. Prolonged exposure to low α-chaconine concentrations or shorter exposure to higher, cytotoxic concentrations of α-chaconine resulted in reduced induction of those genes (*11*). These observations were interpreted to suggest that α-chaconine may have induced indirectly, through the formation of glycoalkaloid/sterol complexes and disturbances in cellular cholesterol levels, the expression of cholesterol biosynthesis genes. This induction may be a response of the cells to keep cholesterol homeostasis.

Cholesterol is an important component of cellular membranes; thus, levels of cholesterol are tightly regulated. Cholesterol biosynthesis genes are regulated at the transcriptional level and their transcriptional regulation is controlled by membrane-bound transcription factors, sterol regulatory element binding proteins (SREBPs) (*14*). SREBPs bind to sterol regulatory elements (SREs) found in the promoter regions of many cholesterol and fatty acid biosynthesis genes inducing their transcription.

The regulation of SREBP activity occurs at the transcriptional and post-translational levels (*15, 16*). The post-translational regulation of SREBP activity involves sterol-mediated suppression of SREBP proteolytic cleavage (*17*). Whereas regulation at the transcriptional level is more complex, one mechanism involves feed-forward regulation, whereby the SREBPs regulate the transcription of their own genes via SRE in the enhancer or promoter region of each gene (*15, 17*). Other factors such as liver X-activated receptors (LXRs), insulin, and glucagon have been shown to regulate SREBP transcription (*15*).

Previously, we observed that exposure of Caco-2 cells to α-chaconine resulted in increased expression of genes involved in phosphatidylinositol 3-kinase (PI3K)/v-akt murine thymoma viral oncogene homologue (AKT), mitogen-activated protein kinase (MAPK), and growth (which are mediated by either AKT or MAPK) signaling pathways and downstream effectors of these pathways, such as Jun, Fos, and c-Myc (*11, 12*). The MAPK family consists of at least three different subgroups that include ERK1/2 (extracellular signal related kinase), JNK (c-jun N terminal protein kinase, also referred to as stress-activated protein kinase, SAPK), and p38. MAP kinases play a pivotal role in orchestrating intracellular events essential for cell functioning, growth, and apoptosis (*18*). On the other hand, PI3K and its substrate, that is, AKT kinases, play a central role in diverse signaling cascades that regulate cell proliferation and survival, cell size, and response to nutrient availability, glucose metabolism, cell invasiveness, genome stability, and angiogenesis (*19*). Once activated, these kinases can phosphorylate and activate transcription factors, which regulate gene expression. Studies demonstrate that in particular the PI3K/AKT pathway is involved in the regulation of SREBP activity at the transcriptional and post-translational levels. AKT activation leads to upregulation of SREBP (*20*) or alternatively induces ER-to-Golgi transport of SREBP and sterol regulatory element binding transcription factor cleavage-activating protein (SCAP), resulting in proteolytic cleavage of SREBP (*21*). On the other hand, the MAPK pathway was shown to regulate the transcriptional activity of SREBPs via phosphorylation of ERK (*22, 23*).

In an attempt to further understand the mechanism by which cholesterol biosynthesis gene transcription is increased in intestinal epithelial cells following exposure to potato glycoalkaloids, in the present study, we investigated the effect of α-chaconine on SREBP-2 protein levels and determined whether the PI3K/AKT or MAPK signaling pathways are necessary for the induced expression of these genes.

MATERIALS AND METHODS

Reagents. α-Chaconine was obtained from Sigma Aldrich (St. Louis, MO). Chemical inhibitors LY294002 (PI3K inhibitor), PD98059 (MEK1 inhibitor), and SP600125 (JNK inhibitor) were purchased from Calbiochem (Darmstadt, Germany). Stock solutions of α-chaconine and the chemical inhibitors were prepared in dimethyl sulfoxide (DMSO) (Merck, Germany). The stock solutions were diluted with Dulbecco's modified Eagle's medium (DMEM) to the final desired concentrations immediately before use. In every experiment, cells in the control group were treated with an equivalent concentration of the solvent (0.01 v/v % DMSO). Rabbit polyclonal antibodies against total p44/42 (ERK), phopho-p44/42 (ERK 1/2) (Thr 202/Tyr 204), phopho-AKT (Ser 473), total AKT, phopho-SAPK/JNK 1/2 (Thr 183/ Tyr 185), total SAPK/JNK, and total p38 were obtained from Cell Signaling (Beverly, MA). Rabbit polyclonal antibody against phospho-p38 (Thr 180/Tyr 182) and goat polyclonal antibody against Actin (C-11) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA), whereas the rabbit polyclonal antibody against SREBP-2 was obtained from Abcam (Cambridge, United Kingdom). The horseradish peroxidase-conjugated antirabbit and donkey antigoat antibodies were obtained from Promega (Madison, WI). Reagents for electrophoresis and Western blotting were obtained from Amersham Bioscience (Arlington Heights, IL). A detergent compatible (DC) protein assay for protein quantification was obtained from BioRad (Hercules, CA).

Caco-2 Cell Culture. The human intestinal epithelial cell line Caco-2 (ATCC, Manassas, VA) was grown routinely in 75 cm^2 culture flasks at 37 °C in air with 5% CO_2 and 100% relative humidity in DMEM (BioWhittaker, Verviers, Belguim) supplemented with $NaHCO_3$ (3.7 g/l, Sigma), nonessential amino acids (1× NEAA; ICN, Zoetermeer, The Netherlands), fetal calf serum (FCS; 10% v/v; Invitrogen, Breda, The Netherlands), penicillin (5000 U, Sigma), and streptomycin (5 mg/L, Sigma).

Treatment with α-Chaconine and Chemical Inhibitors. Caco-2 cells were seeded at a density of 40000 per cm^2 in 6 well polyester Transwell plates (Costar; 0.4 μm pore size, inserts of 24 mm diameter). The cells were allowed to differentiate by growing them for 19 days. Following differentiation, cells were exposed for 6 h to 10 μM α-chaconine. The exposure time and concentration were based on results from a previous study in which optimal conditions for studying the effect of α-chaconine on gene expression were determined systematically (*11*). When chemical inhibitors were used, cells were pretreated for 24 h with either LY294002 (60 μM), PD98059 (50 μM), SP600125 (60 μM), or a combination of all inhibitors, prior to coincubation with 10 μM α-chaconine for 6 h. As stock solutions were dissolved in DMSO, an equal volume of DMSO (final concentration, 0.01% v/v) was added to the control cells. The media in the upper compartments of the transwells were replaced with DMEM containing 0.01% (v/v) DMSO [i.e., control exposure (with or without inhibitor(s)] or 10 μM α-chaconine [with or without inhibitor(s)]. The media in the lower compartments were replaced with DMEM only.

Western Blotting. *Isolation of SREBP-2 Proteins.* Following treatment of Caco-2 cells for 6 h with and without 10 μM α-chaconine, nuclear and membrane fractions were prepared by a modification of the procedure described by Field et al. (*24*). The precursor form of SREBP-2 is predominantly in the membrane fraction, as it is normally bound to membranes of the endoplasmic reticulum and nuclear envelope. After proteolytic cleavage, the

Table 1. Primers Used for Quantitative Real-Time PCR[a]

gene name only	gene symbol	sequence ID	forward primer (5′−3′)	reverse primer (5′−3′)
cytochrome P450, family 51	CYP51A1	NM_000786	CAACTCAATGAAAAGGTAGCACAGC	TCTGCGTTTCTGGATTGCCTTATAG
3-hydroxy-3-methyl-coenzyme A reductase	HMGCR	NM_000859	TTCCAGAGCAAGCACATTAGCAAAG	GCCAAAGCAGCACATAATTTCAAGC
3-hydroxy-3-methyl-coenzyme A synthase	HMGCS1	NM_002130	ACCGCTGCTATTCTGTCTACTGC	TCTCTATTCTGGTCATTAAGGAAGTCATTC
sterol-C5-desaturase	SC5DL	NM_006918	GTTGGTGCTTACATCCTTTATTTCTTCTG	GCCTGGACAGTAAACTTAATCTCTCG
squalene epoxidase	SQLE	NM_003129	ACGAAGAGCCAGTATCAGAAGAGTATC	AAAGTATGTGAAGCCAAGTTGTATAGGG

[a] All primers were intron-spanning.

mature form is released and enters the nucleus, which is the site of action (*24*). Briefly, following treatment, cells were washed twice with PBS and resuspended in ice-cold buffer A [10 mM HEPES-NaOH (pH 7.4), 1.5 mM MgCl2, 10 mM KCl, 1 mM ethylenediaminetetraacetic acid (EDTA), and 1 mM ethyleneglycoltetraacetic acid (EGTA)]. They were allowed to swell for 30 min. Subsequently, the cells were homogenized by passage through a 22 gauge needle 15 times. First, the homogenate was centrifuged at 1500 rpm for 5 min to obtain a nuclear pellet. The supernatant was centrifuged at 40000 rpm for 45 min to isolate a membrane fraction. Nuclear proteins were extracted from the nuclear pellet with 0.1 mL of buffer C [20 mM HEPES-KOH (pH 7.9), 1.5 mM MgCl2, 25% glycerol, 500 mM NaCl, 1 mM EDTA, and 1 mM EGTA]. Membrane proteins were extracted from the membrane fraction with 0.15 mL of buffer B [125 mM Tris (pH 6.0), 160 mM NaCl, and 1% Triton X-100]. Both fractions were sonicated for 10 s followed by centrifugation in a microcentrifuge for 30 min at 14000 rpm. Protease inhibitors *N*-acetyl-leucyl-leucyl-norleucinal (50 μg/mL), 0.5 mM phenylmethylsulfonyl fluoride (PMSF), pepstatin A (5 μg/mL), leupeptin (10 μg/mL), 1 mM Pefabloc, 10 mM DTT, and aprotinin (2 μg/mL) were added to all buffers used for preparing the cell fractions. All protein isolation procedures were performed at 4 °C. Protein concentrations were determined by the BioRad DC protein assay, and the samples were stored at −80 °C until further use.

Isolation of MAPK Kinase Proteins. Upon treatment, Caco-2 cells were washed twice with ice-cold phosphate-buffered saline (PBS) and lysed in 0.5 mL of lysis buffer [20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM sodium orthovanadate, 1 μg/mL leupeptin, and 1 mM PMSF]. Cell debris was removed by centrifugation at 14000 rpm for 10 min at 4 °C. Protein concentrations in the supernatants were determined by the BioRad DC protein assay, and the samples were stored at −80 °C.

For Western blot analyses, 30 (MAPK/AKT) or 80 μg (SREBP-2) of protein was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE, 12.5% polyacrylamide) before transferring onto a polyvinylidene difluoride (PVDF) membrane (GE Healthcare Bio-Sciences Corp., Piscataway, NJ). The PVDF membrane was blocked with 5% skimmed milk in Tris-buffered saline with 0.05% Tween-20 (TBS-T), pH 7.4, for 1 h at room temperature and probed with rabbit antibodies against total or phospho-p38, total or phospho-ERK1/2, total or phospho-JNK, total or phospho-AKT, SREBP-2, and the goat antibody against actin (1:1,000 dilution) at 4 °C overnight. The membrane was washed and incubated with secondary antirabbit (Promega) or donkey antigoat antibodies (Promega) (1:10000 dilution) coupled to horseradish peroxidase for 1 h at room temperature. Antibody−antigen complexes were then detected using the ECL Plus chemiluminescent detection system according to the manufacturer's instructions (GE Healthcare Bio-Sciences). Band intensities were quantified using the software program Quantity One 1-D analysis software version 4.6.1 (Bio-Rad).

Reprobing the Immunoblots. The immunoblots were soaked in stripping buffer (0.7% 2-mercaptoethanol, 2% SDS, and 62.5 mM Tris, pH 6.8) and incubated at 50 °C for 30 min with gentle shaking. After stripping, the membranes were washed four times for 5 min with TBS-T and then blocked in 5% skimmed milk in TBS-T, pH 7.4 for 1 h at room temperature, followed by probing with the primary and secondary antibodies of interest.

Real-Time Reverse Transcription Polymerase Chain Reaction (RT-PCR) of Cholesterol Biosynthesis Genes. Total RNA from Caco-2 cells was isolated using TriZol reagent (Invitrogen, Breda, The Netherlands) as specified in the manufacturer's instructions. To remove any genomic DNA contaminants, RNA samples were treated with DNase-I RNase free (Promega) followed by phenol/chloroform/isoamylalcohol (25:24:1;v:v:v) and chloroform/isoamylalcohol (24:1;v:v) purification steps. The RNA concentration and purity were determined by measurement of absorbance at 260 and 280 nm using a Nanodrop (Isogen Life Science). Complementary DNA (cDNA) was synthesized from 1 μg of total RNA for each sample using the iScript cDNA synthesis kit, following instructions of the manufacturer (Bio-Rad). Primers for SYBR Green probes were designed with Beacon Designer 5.0 (Premier Biosoft International, Palo Alto, CA) and are shown in **Table 1**. After primer design, all primers were run through the National Center for Biotechnology Information (NCBI) Blast database to check for specificity. PCR amplification and detection were performed with the iQ SYBR Green Supermix and the MyIQ single color real-time PCR detection system (Bio-Rad). Standard curves were constructed for each amplified gene sequence using serial dilutions of a reference sample from the cDNA samples known to induce selected genes significantly. The level of mRNA for each gene was normalized using Ribosomal protein L12 (RPL-12) as a reference gene, which was chosen on the basis of microarray data (data not shown) since it showed similar expression levels for control and α-chaconine-treated groups. To verify the RPL12-normalized results, data also were normalized using a well-accepted reference gene Hypoxanthine phosphoribosyltransferase 1 (HPRT1). The outcome of that analysis was similar (data not shown).

Statistical Analysis. Results are expressed as means ± standard deviation (SD). Comparisons of changes in protein levels among treatment groups were analyzed statistically by the unpaired Student's *t* test (two-tailed). RT-PCR gene analysis was conducted using a two-way analysis of variance (ANOVA) with the factors α-chaconine treatment (two levels: with or without α-chaconine) and inhibitor(s) pretreatment [five levels: no inhibitor(s), ERK pathway inhibitor (PD98059), JNK pathway inhibitor (SP600125), PI3K/AKT pathway inhibitor (LY294002), and combination of all inhibitors]. Ratios of the signal intensities of the gene of interest vs the reference gene were calculated, and these ratio values were log transformed to stabilize the variance. The main effects of α-chaconine treatment, inhibitor(s) pretreatment, and interaction of α-chaconine and inhibitor(s) on gene expression were determined.

The effect of inhibitor(s) pretreatment was split up in four contrasts between the level of "no inhibitor(s)" vs each of the other four levels. A *p* value of less than 0.05 was considered statistically significant.

RESULTS

Effect of α-Chaconine on SREBP-2 Protein Expression and Cleavage. To better understand the mechanism through which the expression of cholesterol biosynthesis genes is increased upon treatment of Caco-2 cells with α-chaconine, we determined the possible effect of this treatment on expression of SREBP-2 at the (post)translational level. Upon exposure of differentiated Caco-2 cells to 10 μM α-chaconine for 6 h, nuclear and membrane fractions were isolated from the cell lysate, and Western blot analysis was conducted using a specific antibody against SREBP-2. Western blotting demonstrated that the level of precursor SREBP-2, but not of mature protein, in the membrane fraction was significantly increased after treatment with α-chaconine (**Figure 1A**). With respect to the abundance

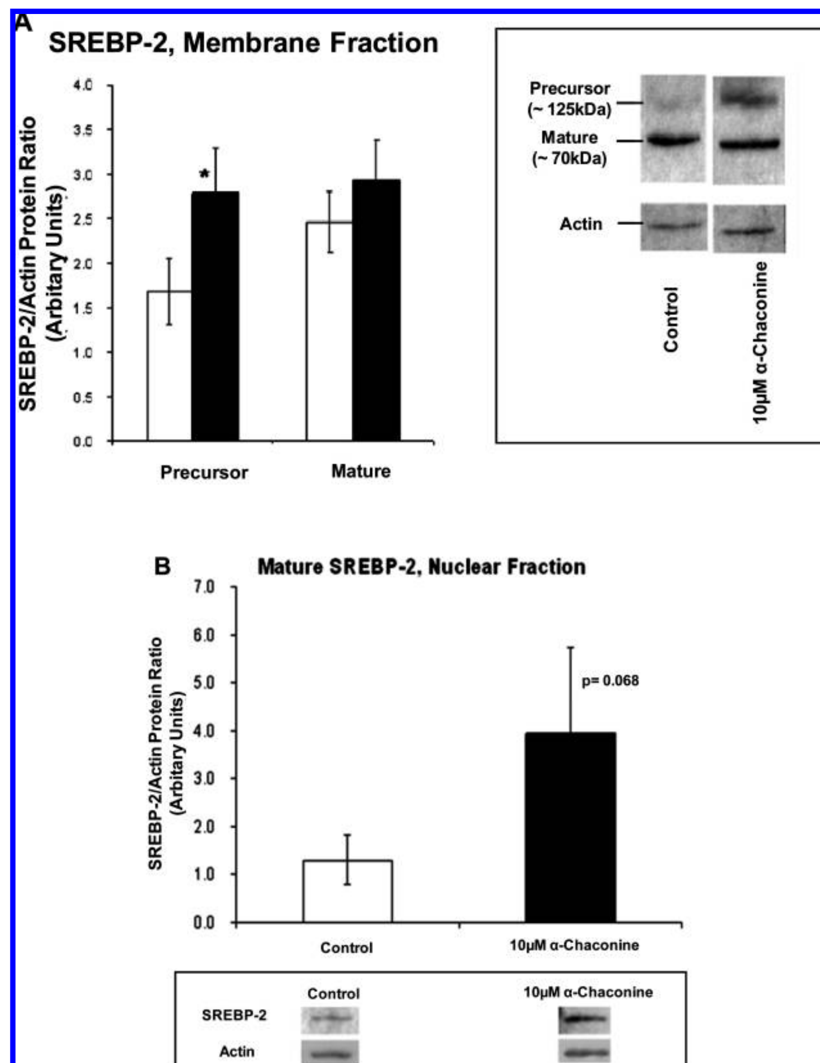

Figure 1. Effect of α-chaconine on SREBP-2 protein expression and cleavage. Caco-2 cells were exposed to 10 μM α-chaconine for 6 h. Membrane (**A**) and nuclear fractions (**B**) (corresponding to 80 μg of protein) were analyzed by Western blotting with an anti-SREBP-2 antibody. The corresponding blots were stripped and reprobed with an actin antibody for assessment of equal loading. Integrated densitometric data from three independent experiments are shown as a bar graph, and the illustrated data represent one of the three independent experiments. Results are presented as means ± SD (n = 3). *Significant difference from solvent control, $p < 0.05$.

of SREBP-2 in the nuclear fraction, no precursor SREBP-2 could be detected (data not shown), but treatment of Caco-2 cells with α-chaconine resulted in a up to 3-fold increase in mature SREBP-2 protein levels (**Figure 1B**). However, this increase was of marginal statistical significance ($p = 0.07$) due to large variation among the α-chaconine-treated samples (SD = ±1.78).

Effect of α-Chaconine on Phosphorylation of MAP and AKT Kinases. Because analysis of data from previous microarray experiments indicated that several genes involved in MAPK and AKT pathways also were affected, we examined by Western blotting whether α-chaconine treatment resulted in activation (i.e., phosphorylation) of AKT and the MAP kinases ERK, JNK, and p38. Exposure of differentiated Caco-2 cells to 10 μM α-chaconine for 6 h resulted in a significant increase in phosphorylation of JNK, ERK, and AKT but not p38 (**Figure 2**). Because p38 was not phosphorylated and thus might not be involved in induction of cholesterol biosynthesis genes by α-chaconine, it was not included in the subsequent kinase inhibitor experiments.

Effects of LY294002, PD98059, and SP600125 on Cholesterol Biosynthesis Gene Expression in the Presence or Absence of α-Chaconine. To examine whether the MAPK and PI3K/AKT pathway phosphorylations induced upon α-chaconine treatment are crucial for the upregulation of cholesterol biosynthesis genes, we determined the effects of cell permeable inhibitors of MEK1 (PD98059), JNK (SP600125), and PI3K (LY294002) on the expression of a number of representative genes by real-time RT-PCR (**Table 2**). LY294002 was used as PI3K as an upstream regulator of AKT (*25*), PD98059 inhibited MEK1, which is a dual-specificity kinase that phosphorylates ERK1/2 (*26*), and SP600125 directly inhibited JNK. Caco-2 cells were pretreated with either PD98059 (50 μM), SP600125 (60 μM), LY294002 (60 μM), or a combination of all inhibitors for 24 h prior to coincubation with 10 μM α-chaconine for 6 h. First, we verified whether the inhibitors were able to block the phosphorylation of ERK, JNK, and AKT. As shown in **Figure 3**, each inhibitor obstructed the phosphorylation of its respective kinase, and α-chaconine could not overcome this inhibition.

Subsequently, RNAs of the treated and nontreated cells were subjected to quantitative RT-PCR. Two-way ANOVA and contrast analysis of the RT-PCR data allowed the determination of the effects of α-chaconine or the inhibitors and possible interaction between the α-chaconine and the inhibitors on the

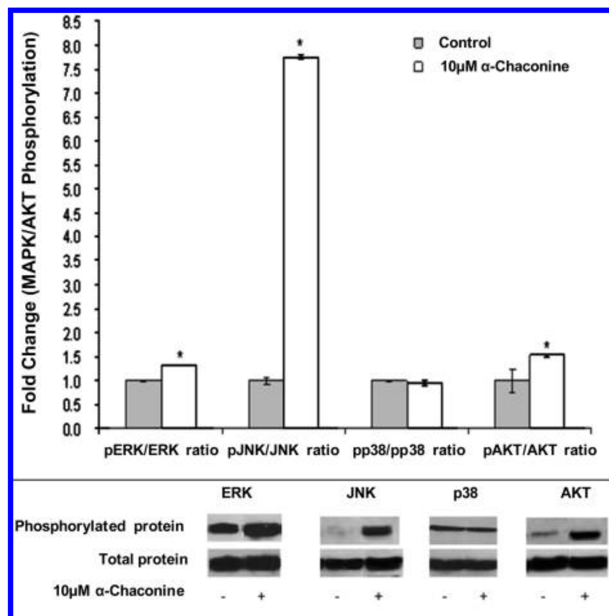

Figure 2. Effect of α-chaconine on ERK, JNK, p38, and AKT phosphorylation. Caco-2 cells were exposed to 10 μM α-chaconine for 6 h. Total cell lysates (30 μg of protein) were analyzed by Western blotting for phosphorylated and total ERK, JNK, p38, and AKT. First, blots were probed for the phosphorylated forms, and then, they were stripped and reprobed with a corresponding antibody against the total protein form for assessment of equal loading. Integrated densitometric data from three independent experiments are shown as a bar graph, and the illustrated data represent one of the three independent experiments. The fold change for each kinase was calculated using the control (basal expression) as the standard (setting value to 1). Results are presented as means + SD ($n = 3$). *Significant difference from solvent control, $p < 0.05$.

transcription of cholesterol biosynthesis genes (**Table 2** and **Figure 4**). In particular, determining the presence of an interaction between the α-chaconine and the kinase inhibitors would reveal the possible role of the MAPK and PI3K/AKT pathways in the induction of cholesterol biosynthesis gene expression following α-chaconine treatment. Our data showed a distinct effect of α-chaconine treatment on the level of cholesterol biosynthesis gene expression, with the exception of SREBP-2. **Figure 4** shows that the expression of most cholesterol biosynthesis genes was increased when cells were exposed to α-chaconine in the absence as well as in the presence of inhibitors (**Table 2**, $p < 0.05$). No significant interaction was observed between α-chaconine treatment and inhibitor(s) pretreatment, except for the SC5DL gene (**Table 2** and **Figure 4**).

For the SC5DL gene, a statistically significant interaction effect was observed between α-chaconine and inhibitor, when all three kinase inhibitors (ERK, JNK, and AKT) were simultaneously applied. A decrease in expression of this gene was observed, suggesting that blocking all pathways affected the induction of this gene (**Table 2**, $p < 0.02$). Kinase inhibitors that were tested, either alone or in combination, however, did not prevent the induction of the other cholesterol biosynthesis genes following α-chaconine exposure. Using the exposures without inhibitors (with or without α-chaconine) as a basis of comparison, pretreatment of the cells with PI3K/AKT pathway inhibitor LY294002 alone or a cocktail of all inhibitors reduced the expression levels of cholesterol biosynthesis genes significantly (**Table 2** and **Figure 4**). On the other hand, pretreatment with the ERK inhibitor PD98059 alone resulted in increased transcription of CYP51A1 (**Figure 4** and **Table 2**, $p < 0.04$). Taken together, the overall outcome of the inhibition experiments indicates that, although particularly the PI3K/AKT signaling pathway is important for basal expression of cholesterol biosynthesis genes, neither this pathway nor JNK and ERK pathways are crucial for the induction of expression of these genes following treatment of Caco-2 cells with α-chaconine.

DISCUSSION

Our previous microarray studies showed that exposure of intestinal epithelial cells to α-chaconine, α-solanine, and varying mixtures of these two glycoalkaloids at noncytotoxic concentrations resulted in increased expression of cholesterol biosynthesis genes (*11, 12*). We also found that genes in the PI3K/AKT and MAPK pathways and their downstream effectors such as Jun, Myc, and Fos were upregulated. To understand the mechanisms underlying this induction of cholesterol biosynthesis genes, the effect of α-chaconine on the expression of SREBP-2, the main regulator of cholesterol biosynthesis, was investigated. Because the MAPK and PI3K/AKT pathways have been shown to regulate the activity of SREBP-2 and consequently cholesterol biosynthesis gene transcription (*20−23*), the importance of PI3K/AKT and MAPK pathways in the induction of cholesterol biosynthesis genes upon α-chaconine treatment also was determined.

Our data indicate that α-chaconine exposure results in changes in SREBP-2 expression at the (post-) translational level. A significant increase in SREBP-2 precursor protein levels in the membrane fraction of the Caco-2 cell lysates was observed. In our previous microarray studies (*11, 12*), we observed a significant increase in SREBP-2 expression following exposure of Caco-2 cells to noncytotoxic concentrations of glycoalkaloids. However, in this study, quantitative RT-PCR analysis did not reveal significantly elevated levels of SREBP-2 transcripts. Therefore, the increase in the amount of precursor protein in α-chaconine-treated cells could be a reflection of increased SREBP-2 protein stabilization, as normally these proteins are rapidly ubiquitinated and degraded by 26S proteosome (*27*) and not due to increased SREBP-2 gene expression. In addition, we also observed a 3-fold increase in mature SREBP-2 protein levels in the nuclear fraction of cells exposed to α-chaconine. This increase of cleaved SREBP-2 in the nucleus is correlated to increased precursor protein levels in the membrane fraction of the cell lysates. However, other explanations for the increase of mature SREBP-2 levels can be envisaged. It might be (at least partially) attributed to a stimulatory effect of α-chaconine on translocation of the mature SREBP-2 fragment into the nucleus, where it activates transcription of cholesterol biosynthesis genes. The initial trigger for this stimulatory effect may be due to depletion of cholesterol in membranes caused by the formation of glycoalkaloid/sterol complexes (*10*). A decrease in the amount of membrane cholesterol may promote a sterol-mediated proteolytic cleavage that increases levels of mature SREBP-2 protein (*18*).

In the current study, Western blot analyses revealed that the levels of phosphorylated AKT, JNK, and ERK proteins increased following treatment of Caco-2 cells with α-chaconine. Subsequently, we determined whether the ERK, JNK, or PI3K/AKT pathways were necessary for the induction of cholesterol biosynthesis genes in cells exposed to α-chaconine using specific kinase inhibitors. We observed a significant increase in expression of cholesterol biosynthesis genes in Caco-2 cells following α-chaconine treatment, which confirmed the results of our previous studies (*11, 12*). Inhibiting the signaling pathways, particularly the PI3K/AKT pathway by LY294002, reduced the (basal) expression levels of cholesterol biosynthesis genes

Table 2. Effects of Kinase Inhibitors LY294002, PD98059, and SP600125 on α-Chaconine-Induced Cholesterol Biosynthesis Gene Transcription[a]

source of variation	*p* values for each gene					
	CYP51A1	HMGCR	HMGCS1	SC5DL	SQLE	SREBP-2
effect of α-chaconine (±inhibitors)	0.007	<0.001	<0.001	0.014	<0.001	0.307
effect of inhibitors (±α-chaconine)						
control (no inhibitors) vs ERK pathway inhibitor (PD98059)	0.039	0.117	0.059	0.231	0.252	0.509
control (no inhibitors) vs JNK pathway inhibitor (SP600125)	0.718	0.672	0.918	0.105	0.836	0.509
control (no inhibitors) vs AKT pathway inhibitor (LY294002)	0.020	<0.001	<0.001	<0.001	<0.001	0.002
control (no inhibitors) vs all pathways inhibitors	0.036	<0.001	<0.001	<0.001	<0.001	<0.001
interaction effect: inhibitor · α-chaconine						
control (no inhibitors) · α-chaconine vs ERK pathway inhibitor · α-chaconine	0.608	0.720	0.814	0.066	0.563	0.110
control (no inhibitors) · α-chaconine vs JNK pathway inhibitor · α-chaconine	0.319	0.905	0.854	0.319	0.871	0.774
control (no inhibitors) · α-chaconine vs AKT pathway inhibitor · α-chaconine	0.629	0.717	0.262	0.096	0.641	0.818
control (no inhibitors) · α-chaconine vs all pathways inhibitors · α-chaconine	0.663	0.591	0.538	0.016	0.792	0.443

[a] Caco-2 cells were incubated with PD98059 (50 μM), SP600125 (60 μM), LY294002 (60 μM), or all chemical inhibitors for 24 h prior to exposure to 10 μM α-chaconine for 6 h. Effects of the chemical inhibitors on cholesterol biosynthesis gene transcription were analyzed by real-time RT-PCR. RPL12 was used as an internal reference. A two-way ANOVA and contrast analysis was conducted to determine significance of the effects of α-chaconine and the inhibitors on gene expression.

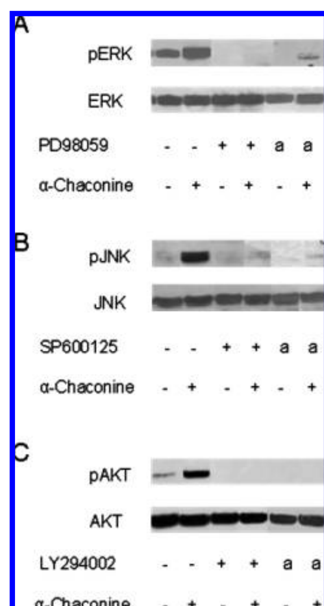

Figure 3. Confirmation of the inhibition of phophorylation of ERK, JNK, and AKT kinases by specific chemical inhibitors. Caco-2 cells were incubated with PD98059 (50 μM; **A**), SP600125 (60 μM; **B**), LY294002 (60 μM; **C**), or all chemical inhibitors (indicated with "a" in panels **A**−**C**) for 24 h prior to exposure to 10 μM α-chaconine for 6 h. Total cell lysates (30 μg of protein) were analyzed by Western blotting for phosphorylated and total ERK, JNK, and AKT. First, blots were probed for the phosphorylated forms, and then, they were stripped and reprobed with a corresponding antibody against all forms for assessment of equal loading. The illustrated data represent one of the three independent experiments.

significantly, in both the absence and the presence of α-chaconine. Du et al. (*21*) demonstrated that inhibiting the activation of AKT in green fluorescent protein (GFP)-SCAP expressing cells by either blocking PI3K (LY294002) or transfection of a plasmid encoding a dominant-negative form of AKT results in disruption of ER-to-Golgi transport of GFP-SCAP and inhibition of SREBP processing. Portsmann et al. (*20*) also demonstrated that activation of SREBP by AKT leads to the induction of key enzymes of the cholesterol and fatty acid biosynthesis pathways and, thus, membrane lipid biosynthesis. Our study is in agreement with these findings, indicating the role of the PI3K/AKT pathway in cholesterol biosynthesis gene regulation. Our studies further elaborate the significance of AKT is maintaining basal levels of cholesterol biosynthesis gene expression in intestinal epithelial cells, consequently cholesterol homeostasis.

In spite of these inhibition effects, two-way ANOVA analysis revealed no significant interactions between the α-chaconine and the inhibitors of ERK, JNK, or PI3K/AKT pathways, indicating that the inhibitors and α-chaconine did not influence each other's effect on the transcription of cholesterol biosynthesis genes, except for SC5DL. Exposure of the cells to all three inhibitors resulted in a decrease in basal SC5DL transcript levels, but unlike for most of the other cholesterol biosynthesis genes studied, these levels were further reduced upon simultaneous exposure to α-chaconine. It is difficult to give an explanation for this different behavior of SC5DL. Little is known on the regulation of this gene and the function of its product, sterol-C5-desaturase, in human (intestinal) cells. The main role of this enzyme is in the cholesterol biosynthesis pathway where it catalyzes the conversion of lathosterol into 7-dehydrocholesterol. Mutations in SC5DL have been associated with lathosterolosis, which is an inborn multiple-malformation/mental retardation syndrome (*28*). Given that five out of the six genes analyzed did not show a significant interaction effect between inhibitors and α-chaconine, we conclude that activation of these pathways is not absolutely required for the induction of cholesterol biosynthesis gene expression in α-chaconine-treated Caco-2 cells but may have a modulatory role (i.e., enhancer effect). The results suggest that the induction of cholesterol biosynthesis gene expression occurs through other more pivotal mechanisms, which may be independent of the MAPK and PI3K/AKT pathways. We observed that α-chaconine treatment resulted in an increase of precursor and mature SREBP-2 in the membrane and nuclear fractions of Caco-2 cell lysates, respectively. It still remains to be determined whether these changes in the level of SREBP-2 are linked to a mechanism that is crucial for the induction of cholesterol biosynthesis gene expression upon α-chaconine treatment and acts independently of the MAPK and PI3K/AKT pathways.

Thus far, the precise mechanisms underlying the toxicological and potential beneficial effects of glycoalkaloids are poorly understood. As indicated in the present study, the PI3K/AKT, JNK, and ERK signaling pathways were altered in cells exposed to α-chaconine and may play a role in some of the observed effects. It is likely that the biological processes affected by these pathways are dependent on the cell type, glycoalkaloid concentration, and the status of other signal transduction pathways. For instance, Yang and co-workers (*29*) observed that in HT29 cells, α-chaconine induced apoptosis, which may be mediated through the suppression of ERK1/2 phosphorylation and subsequent activation of caspase 3, whereas we have observed increased phosphorylation of ERK1/2.

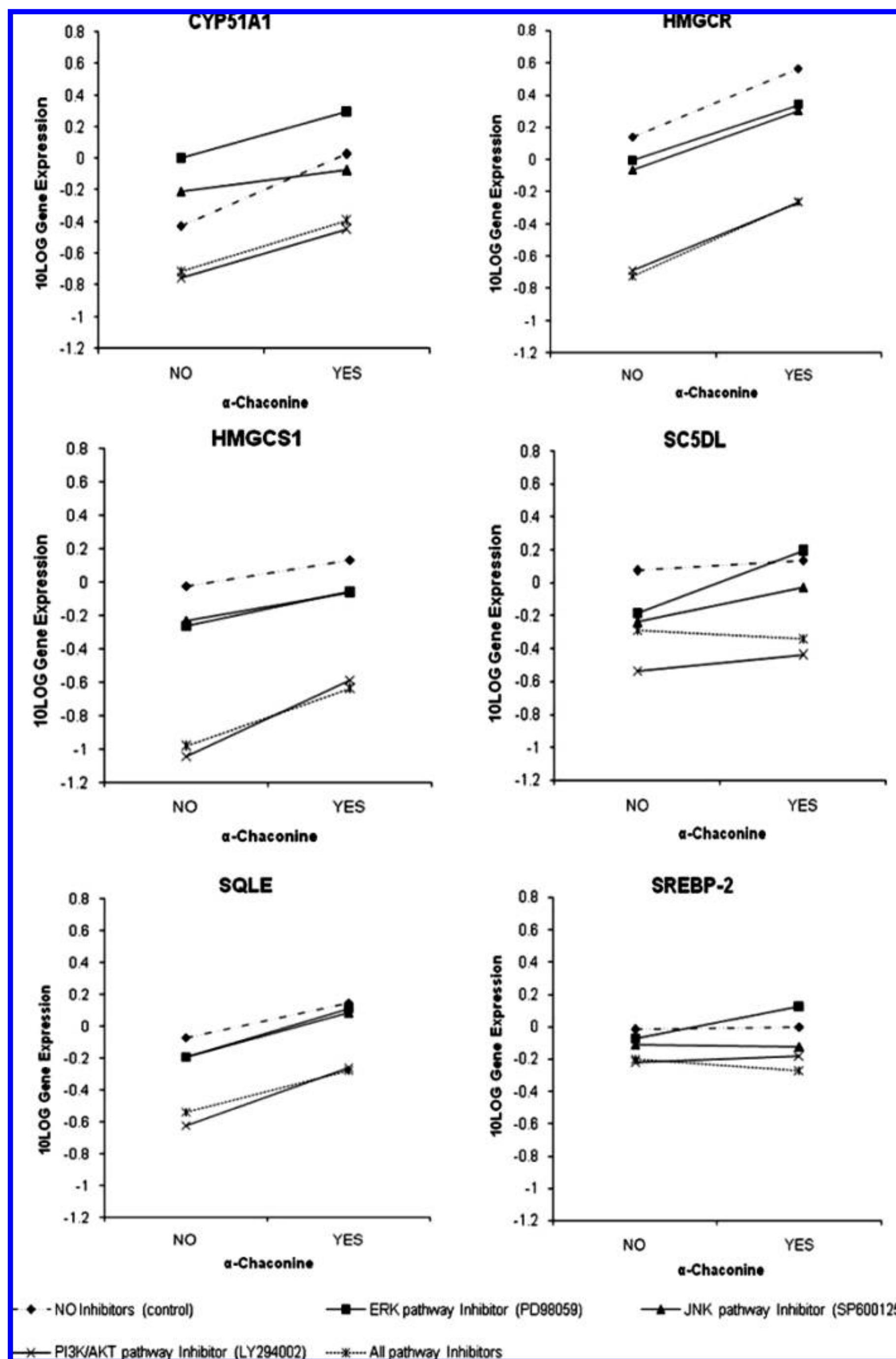

Figure 4. Two-way ANOVA to examine possible interaction of α-chaconine and inhibitors [ERK pathway inhibitor (PD98059), JNK pathway inhibitor (SP600125), PI3K/AKT pathway inhibitor (LY294002), and combination of all inhibitors] on expression of selected cholesterol biosynthesis gene.

In conclusion, this study indicates that exposure of intestinal epithelial cells to α-chaconine induces phosphorylation of AKT, ERK, and JNK. However, these phosphorylation events are not necessary for the observed induction of cholesterol biosynthesis genes. Because these signaling pathways play a central role in many diverse cellular processes and possibly influence glycoalkaloid toxic outcomes, further studies of their roles in other glycoalkaloid-related effects, for example, apoptosis and cell cycle effects (*11, 12*), would be informative. The present work contributes to a better understanding of the molecular mechanisms of action of glycoalkaloids and may lead to a better understanding of their toxic and possible beneficial effects. Moreover, the results of this work may possibly provide insight into more general mechanisms that a cell employs to cope with membrane disruption.

ABBREVIATIONS USED

AKT, v-akt murine thymoma viral oncogene homologue; DMEM, Dulbecco's modified Eagle's medium; DMSO, dimethyl sulfoxide; ERK, extracellular signal-related protein kinase; JNK, c-jun N terminal protein kinase; MAPK, mitogen-activated protein kinase; PI3K, phosphatidylinositol 3-kinase; SRE, sterol regulatory element; SREBP, sterol regulatory element binding transcription factor; SCAP, sterol regulatory element binding transcription factor cleavage-activating protein.

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