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Activation of Bile Acid Biosynthesis by the p38 Mitogen-activated Protein Kinase (MAPK):

HEPATOCYTE NUCLEAR FACTOR-4α PHOSPHORYLATION BY THE p38 MAPK IS REQUIRED FOR CHOLESTEROL 7α-HYDROXYLASE EXPRESSION*

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Abstract

Bile acids are required for intestinal absorption and biliary solubilization of cholesterol and lipids. In addition, bile acids play a crucial role in cholesterol homeostasis. One of the key enzymes in the bile acid biosynthetic pathways is cholesterol 7*α*-hydroxylase/cytochrome P450 7*α*-hydroxylase (7*α*-hydroxylase), which is the rate-limiting and regulatory step of the “classic” pathway. Transcription of the 7*α*-hydroxylase gene is highly regulated. Two nuclear receptors, hepatocyte nuclear factor 4*α* (HNF-4*α*) and α_1-fetoprotein transcription factor, are required for both transcription and regulation by different physiological events. It has been shown that some mitogen-activated protein kinases, such as the c-Jun N-terminal kinase and the ERK, play important roles in the regulation of 7*α*-hydroxylase transcription. In this study, we show evidence that the p38 kinase pathway plays an important role in 7*α*-hydroxylase expression and hence in bile acid synthesis. Inhibition of p38 kinase activity in primary hepatocytes results in ~5–10-fold reduction of 7*α*-hydroxylase mRNA. This suppression is mediated, at least in part, through HNF-4*α*. Inhibition of p38 kinase activity diminishes HNF-4*α* nuclear protein levels and its phosphorylation *in vivo* and *in vitro*, and it renders a less stable protein. Induction of the p38 kinase pathway by insulin results in an increase in HNF-4*α* protein and a concomitant induction of 7*α*-hydroxylase expression that is blocked by inhibiting the p38 pathway. These studies show a functional link between the p38 signaling pathway, HNF-4*α*, and bile acid synthesis.

Cholesterol is an important precursor of several compounds that provide key physiological requirements of mammals. However, excessive accumulation of cholesterol is a risk factor for cardiovascular diseases, which constitute a major health issue. Bile acid synthesis in the liver is the predominant pathway for cholesterol catabolism. Cholesterol 7*α*-hydroxylase/cytochrome P450 7*α*-hydroxylase (7*α*-hydroxylase)[2] is the rate-limiting enzyme in the

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conversion of cholesterol to bile acids and is a key target for the negative feedback regulatory effects of bile acids on their own synthesis.

Several nuclear receptors play key roles in the bile acid biosynthetic pathways. Among them are hepatocyte nuclear factor 4α (HNF-4α) and α_1-fetoprotein transcription factor (FTF), also known as liver receptor homologue 1. HNF-4α is an important regulator of 7α-hydroxylase gene expression as demonstrated by *in vitro* promoter analysis (1) and by characterization of mice with a liver-specific deficiency of HNF-4α (2) or FTF deficiency (3). HNF-4α is a liver-enriched and highly conserved member of the nuclear receptor superfamily (NR2A1). Amino acid identity between rat and human HNF-4 varies from 89.7 to 100%, depending on the isoform. It binds as a homodimer to direct repeats separated by one nucleotide (DR1) response element with the consensus sequence: AGGTCAGGGG(T/A)CA (4). HNF-4α can activate gene transcription in the absence of exogenous ligands. HNF-4α binding activity may be modulated by fatty acyl-coenzyme A thio-esters, which may act as agonistic or antagonistic ligands depending on chain length and degree of saturation (5, 6). The expression of a wide variety of genes is under the control of HNF-4α; among them are genes involved in lipid metabolism, glucose metabolism, and overall development (7).

In the last few years, it has become clear that bile acids are also important regulatory molecules, not only of their own synthesis but also of cholesterol synthesis (8), gluconeogenesis (9), glycogenesis (10), and apoptosis. Many of these regulations occur through the modulation of different cell signaling pathways. Moreover, different cell signaling pathways regulate bile acid synthesis differently. Bile acids can activate both the c-Jun N-terminal kinase (JNK) and the extracellular receptor kinase (ERK) in hepatocytes (11–13). This bile acid-mediated induction of the JNK pathway is part of the mechanism by which bile acids regulate their own synthesis. Thus, the JNK pathway has been shown to activate the small heterodimer partner expression through an farnesoid X-activated receptor-independent pathway (11) in primary hepatocytes. Other cell signaling pathways have also been implicated in the feedback regulation of bile acid synthesis. Thus, bile acids can repress 7α-hydroxylase promoter activity by reducing the transactivation potential of HNF-4α via the MAPK/ERK kinase kinase 1 signaling cascade (14). Furthermore, interleukin-1β inhibits the expression of another important gene in bile acid synthesis, sterol 12α-hydroxylase/CYP8B1, via the JNK pathway (15), presumably by phosphorylating HNF-4α and preventing binding to its recognition site in the sterol 12α-hydroxylase promoter.

The MAPK family of signaling pathways consist of a cascade of sequentially acting upstream kinases: MAPK kinase kinases and MAPK kinases, and at least four terminal kinases: the classic p42/44 MAPK or ERK1/2; JNK1/2; p38 MAPK (p38 kinase); and ERK5. MAPKs phosphorylate a number of transcription factors known to alter their transactivating potentials, thus presumably influencing gene expression to elicit a cellular response. Specific to bile acid biosynthesis, it has been reported that bile acids inhibit 7α-hydroxylase gene transcription and HNF-4α transactivating potential via activating the JNK/c-Jun pathway (14). This suggests that MAPK phosphorylation of nuclear receptors may be an important regulatory mechanism for bile acid metabolism. Phosphorylation of nuclear receptors provides an important mechanism for cross-talk between signaling pathways and has been shown to modulate the activity of many nuclear receptors (16).

[2]The abbreviations used are: 7α-hydroxylase, cholesterol 7α-hydroxylase; FTF, α_1-fetoprotein transcription factor; HNF, hepatocyte nuclear factor; MAPK, mitogen-activated protein kinase; JNK, c-Jun N-terminal kinase; ERK, extracellular signal-regulated kinase; PGC-1α, peroxisome proliferator-activated receptor γ coactivator 1α.

There are several reports indicating that HNF-4α is phosphorylated and that phosphorylation has a functional role. Thus, HNF-4α has been shown to be phosphorylated by the action of protein kinase A, and this phosphorylation modulates its DNA binding activity (17). Serine/threonine phosphorylation of HNF-4α alters its tertiary structure, which causes an increase in the affinity and specificity of DNA binding in COS-7 cells (18). It has also been recently shown that p38 kinase phosphorylates HNF-4α, increasing its DNA binding and transactivation activity in the presence of interleukin-1β + H_2O_2 (19). On the other hand, tyrosine phosphorylation of HNF-4α is also important for DNA binding activity as well as for the recruitment of its transcriptionally active form into subnuclear compartments (20).

In this study, we show evidence that p38 kinase plays an important role in 7α-hydroxylase expression and hence in bile acid synthesis. Studies are shown that indicate that the effect of p38 kinase is mediated, at least in part, through HNF-4α. Inhibition of p38 kinase activity diminishes HNF-4α phosphorylation *in vivo* and renders a less stable protein. Insulin, a known activator of the p38 kinase pathway, increases nuclear HNF-4α protein levels, which results in an induction of 7α-hydroxylase expression. These studies show a functional connection between the p38 signaling pathway and a key liver nuclear receptor, HNF-4α.

EXPERIMENTAL PROCEDURES

Materials and Plasmids

p38 kinase inhibitors, SB 202190, and SB 203580 and the nonactive chemical analogue SB 202474 were obtained from CalBiochem. Monoclonal Anti-Myc antibody was from Sigma. Anti-HNF-4α and anti-histone deacetylase 1 antibodies were obtained from Santa Cruz Biotechnology. Anti-phospho-p38 kinase and anti-p38 kinase were obtained from Cell Signaling Technology. [^{32}P]Orthophosphate was from MP Biomedicals, Inc (Irvine, CA). L-[^{35}S]Methionine was from PerkinElmer Life Sciences. p38β2/SAPK2b2 was from Upstate Biotechnology, Inc. (Lake Placid, NY). Common laboratory chemicals were from Fisher, Sigma, or Bio-Rad. The chimeric 7α-hydroxylase promoter-luciferase reporter construct pGL3-R7α-342 has been described previously (21).

Primary Rat Hepatocyte Cultures

Primary rat hepatocyte monolayer cultures were prepared from male Harlan Sprague-Dawley rats (200–300 g) using the collagenase perfusion technique of Bissell and Guzelian as previously described (21). Hepatocytes were plated on collagen-coated culture dishes in serum-free William's E medium containing 0.1 μM dexamethasone and 1 μM thyroxine. Before plating, the cells were judged to be greater than 90% viable using trypan blue exclusion. The cells were routinely incubated at 37 °C in humidified 5% CO_2. When treated with p38 kinase chemical inhibitors, a final concentration of 5 μM was used, delivered in Me_2SO.

RNA Isolation and Quantification

Total RNA was isolated using the SV total RNA isolation kit (Promega) according to the manufacturer's protocol. 7α-Hydroxylase, HNF-4α, and β-actin mRNAs were quantified by quantitative reverse transcription PCR using the primers and probes shown in Table 1 and the Brilliant quantitative reverse transcription PCR Core Reagent kit (Stratagene) using either TaqMan probes (7α-hydroxylase) or SYBR Green (HNF-4α) with a DNA Engine Opticon2 (MJ Research, Inc.). All of the values were normalized to the levels of actin mRNA, also quantified by quantitative reverse transcription PCR either in separate SYBR Green reactions or in duplex reactions when TaqMan probes were used.

Bile Acid Synthesis

Bile acid synthesis rates were quantified essentially as described (22). Briefly, primary rat hepatocytes were seeded on 60-mm plates at 2.5×10^6 cells/plate. Twenty-four hours later the cells were incubated in the presence or absence of the p38 kinase inhibitors as indicated for 2 h. Then 0.13 μCi of [^{14}C]cholesterol (50 mCi/mmol) was added per plate and incubated for 48 h. Conversion of [^{14}C]cholesterol to [^{14}C]methanol/water-soluble products was determined by scintillation counting after Folch extraction (23) of the culture medium with chloroform/methanol (2:1, v/v). Where indicated, the cells were infected at a multiplicity of infection of 40 of an adenovirus encoding the steroidogenic acute regulatory domain 1 cDNA driven by the cytomegalovirus promoter (24) in 0.5 ml of serum-free medium. After 2 h, the virus was diluted, and [^{14}C]cholesterol was added and processed as above.

Western Blot Analysis

Primary rat hepatocyte cytoplasmic and nuclear extracts were prepared using a nuclear and cytoplasmic kit from Pierce. 10 μg of nuclear protein or 60 μg of cytoplasmic protein was fractionated on an SDS-polyacrylamide gel, transferred to nitrocellulose; incubated with the anti-HNF-4α, anti-histone deacetylase 1, or anti-P-p38, or anti-total p38; and processed with a Western Lightning Chemiluminescence Reagent Plus kit (PerkinElmer Life Sciences) according to the manufacturer's recommendations.

Transient Transfection and Luciferase Assays

Primary rat hepatocytes were transfected with Lipofectin (Invitrogen) using 1.5 μg of total DNA containing 100 ng of pGL3-R7α-342 (wild type) and 10 ng of pCMV-Gal to normalize for transfection efficiencies. After 16 h the DNA was removed, and p38 kinase inhibitors were added. The cells were harvested 24 h later. CV-1 cells were first infected with the indicated virus for 7 h and then transfected with pGL3-R7α-342 using FuGENE 6 reached (Roche Applied Science). After transfection the indicated p38 chemical inhibitors were added to 1% fetal calf serum containing medium. Luciferase and β-galactosidase assays were performed with a kit from Applied Biosystems (Foster City, CA), according to the manufacturer's protocol. All of the transfections were performed in duplicate. The values are averages for the number of experiments indicated.

Adenovirus Preparation and Propagation

The adenovirus Ad-Myc-HNF-4α was obtained through the Massey Cancer Center Shared Resource Facility of the Virginia Commonwealth University as described (3). The dominant negative kinase-deficient mutant p38 kinase was a gift from Dr. P. Dent (25).

Metabolic Labeling

Primary rat hepatocytes plated on 60-mm dishes were infected with multiplicity of infection of 10 of the Ad-Myc-HNF-4α adenovirus. After overnight incubation with the virus, the medium was replaced with fresh William's E medium. Two hours later, the cells were washed with phosphate-free Dulbecco's modified Eagle's medium and treated with p38 kinase inhibitors. After 30 min of incubation, the cells were labeled with 1 mCi of [^{32}P]orthophosphate for 2 h. The cells were then lysed in 500 μl of radioimmune precipitation assay buffer (20 mM Tris, pH 8.0, 150 mM NaCl, 1% Nonidet P-40, 0.1% SDS, 20 mM NaF, 2 mM EDTA, 1 mM sodium vanadate, and proteinase inhibitors). The cell lysates were incubated with 2 μg of anti-Myc antibody or preimmune IgG as control at 4 °C overnight, and then the immune complexes were precipitated by incubating the samples with protein A/G-agarose (Sigma) at 4 °C overnight. Washed beads were boiled in 50 μl of Laemmli buffer and loaded onto the SDS-PAGE gel. The gel was subjected to

autoradiography to detect phosphorylated proteins or Western blotting analysis using anti-HNF-4α antibody to detect the protein. Studies of HNF-4α protein stability were similarly conducted in primary rat hepatocytes. Primary rat hepatocytes were infected with a multiplicity of infection of 10 of the Ad-Myc-HNF-4α adenovirus overnight. The medium was then changed to L-methionine, starved for 30 min, and then labeled with 120 μCi/ml L-[^{35}S]methionine (PerkinElmer Life Sciences) for 40 m. The cells were then washed and incubated in regular Dulbecco's modified Eagle's medium with or without 5 μM of the p38 kinase inhibitors. Samples were harvested for immunoprecipitation with the anti-Myc antibody at different intervals. Measurement of the incorporation of L-[^{35}S]methionine into immunoprecipitable Myc-HNF-4α was carried out by scintillation counting.

Statistical Analysis

The results are expressed as the means ± S.D. Statistical analysis was performed using the SPSS 11 software. Comparisons in groups were analyzed by using the *t* test or one-way analysis of variance; $p \leq 0.05$ was considered significant.

RESULTS

Decreased 7α-Hydroxylase Expression in Primary Rat Hepatocytes Treated with p38 Kinase Inhibitors

To investigate whether the p38 MAPK pathway plays a role in 7α-hydroxylase expression and bile acid biosynthesis, we incubated primary rat hepatocytes with two specific chemical inhibitors of the p38 kinase, SB 202190 and SB 203580. As a control we used the inactive analogue SB 202474. Fig. 1*A* shows that both p38 kinase inhibitors decreased 7α-hydroxylase mRNA between 80 and 90% as compared with controls. To confirm the specificity of this effect, we used a genetic approach to suppress p38 kinase activity. We used a dominant negative kinase-deficient mutant p38 kinase expressing virus (25). Fig. 1*B* shows a dose-dependent suppression of 7α-hydroxylase mRNA by the p38 mutant but not by a control virus. Actin mRNA was quantified as control and used to normalize 7α-hydroxylase mRNA values.

The lower 7α-hydroxylase expression caused by the p38 inhibitors resulted in lower bile acid synthesis in the hepatocytes as quantified by measuring bile acids excreted in the medium (Fig. 2). Bile acid levels inside the cells showed a decreasing trend, although it was statistically insignificant. As a positive control we used cells overexpressing steroidogenic acute regulatory domain protein 1, which we have shown to increase bile acid synthesis (24). Bile acids excreted in the medium of steroidogenic acute regulatory domain protein 1-infected cells increased 11-fold compared with control (data not shown).

To characterize whether p38 kinase inhibitors suppressed 7α-hydroxylase expression at the transcriptional level, we transfected primary hepatocytes with a 7α-hydroxylase promoter luciferase construct containing 342 bp of the rat 5′-flanking region (21). After transfection, the cells were treated with the same p38 kinase inhibitors as above. Fig. 3*A* shows that 7α-hydroxylase promoter activity was suppressed by the p38 kinase inhibitors in the same manner as the 7α-hydroxylase mRNA. Then we used a non-liver cell line (CV-1) that does not express the 7α-hydroxylase promoter unless exogenous factors are cotransfected (26). Transactivation of the 7α-hydroxylase promoter by HNF-4α is specifically suppressed by the p38 kinase inhibitor SB 202190 but not by the inactive analogue SB 202474 (Fig. 3*B*), suggesting that HNF-4α mediates, at least in part, the p38 activation of 7α-hydroxylase expression.

Decreased Nuclear HNF-4α Protein Levels in Primary Rat Hepatocytes Treated with p38 Kinase Inhibitors

To characterize whether HNF-4*α* mediates the p38 kinase effect on 7*α*-hydroxylase expression, we quantified HNF-4*α* protein in hepatocyte nuclear extracts that had been treated with the same p38 kinase inhibitors. Fig. 4*A* shows that the nuclear HNF-4*α* protein was reduced in hepatocytes treated with the specific p38 kinase inhibitors (SB 202190 and SB 203580) for 18 h. Histone deacetylase 1 was quantified as a control, and it was unchanged. Levels of FTF protein, another key transcriptional factor for 7*α*-hydroxylase expression, did not change (data not shown). However, HNF-4*α* mRNA was unchanged (Fig. 4*B*), suggesting that p38 kinase affects HNF-4*α* translation or protein stability. To address this issue, pulse-chase experiments were carried out in primary rat hepatocytes infected with an adenovirus to overexpress HNF-4*α* in the form of a fusion protein Myc-HNF-4*α* to facilitate immunoprecipitation. Then proteins were metabolically labeled with [^{35}S]Met, incubated with p38 inhibitors and processed as indicated under “Experimental Procedures.” Fig. 5 shows the average of three experiments, and it illustrates that the half-life of the HNF-4*α* protein significantly decreased when cells had been incubated in the presence of the specific p38 inhibitor SB 203580.

p38 Kinase Phosphorylates HNF-4α in Primary Rat Hepatocytes

To study whether HNF-4*α* is phosphorylated in primary rat hepatocytes, we infected cells with the same adenovirus containing a Myc-HNF-4*α* fusion cDNA as above. After overnight overexpression of the fusion protein, the cells were incubated with [^{32}P]phosphate in the presence of the p38 kinase inhibitors SB 202190 and SB 203580, or a control inhibitor (SB 202474), or just the vehicle (Me_2SO). After 2 h of incubation, the cells were harvested and processed as indicated under “Experimental Procedures.” Fig. 6 shows that HNF-4*α* is indeed phosphorylated and that phosphorylation is prevented by the p38 kinase inhibitors by ~40%. The Myc-HNF-4*α* protein remained constant under all conditions. This is in agreement with a very recent publication showing that HNF-4*α* is phosphorylated in HepG2 cells (19). It is also in agreement with HNF-4*α* being phosphorylated by other kinases (17, 18), because p38 kinase inhibitor prevented phosphorylation only by a factor of 2.

p38 Activation by Insulin Increases HNF-4α Protein and 7α-Hydroxylase Expression

Insulin has been described as activating the p38 pathway in kidney cells (27). Insulin is also a known regulator of bile acid biosynthesis. Thus, recently it has been shown that short term treatment of human primary hepatocytes with physiological concentrations of insulin activates 7*α*-hydroxylase expression, whereas longer incubation suppresses it (28). Thus, to investigate whether the p38 kinase pathway could be involved in this regulatory process by increasing nuclear HNF-4*α* protein levels, we incubated primary rat hepatocytes with different insulin concentrations at physiological range (1 to 10 nM). Fig. 7*A* shows that 7*α*-hydroxylase expression increases at the concentrations tested after 6 h but not at shorter times (2 h). Longer incubation (20 h) had either no effect or resulted in a suppression at the highest insulin concentration tested (10 nM), as it has been reported in human hepatocytes (28). HNF-4*α* mRNA remained unchanged at all conditions (Fig. 7*B*). Then we quantified HNF-4*α* nuclear protein and active (phosphorylated) p38 kinase under conditions that result in induction of 7*α*-hydroxylase expression (10 nM for 6 h). Under those conditions, the increase in 7*α*-hydroxylase expression correlated with an increase in nuclear HNF-4*α* protein (Fig. 7*C*), which followed an increase in activated p38 kinase (Fig. 7*D*). This strongly suggests that insulin induces 7*α*-hydroxylase expression by activating the p38 kinase pathway, which results in increased levels of nuclear HNF-4*α* protein, a critical factor for 7*α*-hydroxylase transcription.

To test the specificity of this effect, primary rat hepatocytes were pretreated with the p38 inhibitor SB 203580 and then with insulin. Fig. 8 shows that when the p38 kinase pathway is inhibited, insulin has no significant effect on 7*α*-hydroxylase mRNA (Fig. 8*A*). To test whether the phosphorylation state of HNF-4*α* is altered by insulin, primary rat hepatocytes were infected with the Myc-HNF-4*α* adenovirus overnight, treated with the p38 inhibitor where indicated, labeled with [^{32}P]phosphate, and then incubated with or without 10 nM insulin as indicated. HNF-4*α* phosphorylation significantly increased upon insulin treatment in the absence of the p38 kinase inhibitor SB 203580 (Fig. 8*B*, *lanes 1* and *3*) but not in its presence (Fig. 8*B*, *lanes 2* and *4*). Myc-HNF-4*α* protein levels did not significantly change under the short incubation time used. Taken together, the experiments shown in Figs. 7 and 8 strongly support the idea that the short term activation of 7*α*-hydroxylase expression by insulin is mediated, at least in part, by activating the p38 kinase, which in turn increases HNF-4*α* phosphorylation, which results in higher levels of HNF-4*α* driving increased 7*α*-hydroxylase expression.

DISCUSSION

Studies carried out in the last decade have unveiled two general regulatory pathways of bile acid biosynthesis, *i.e.* a “nuclear receptor” pathway and a “signaling” pathway. Until recently, the two pathways have remained mostly independent of each other as far as bile acid metabolism is concerned. Specifically, there is good evidence that two of the major signaling pathways, *i.e.* the JNK pathway (11) and the ERK pathway (29), are activated by bile acids. Induction of these pathways results in suppression of bile acid synthesis by acting on 7*α*-hydroxylase transcription. In the present study, we provide additional evidence for cross-talk between a nuclear receptor pathway and a signaling pathway, implicating, for the first time, the p38 kinase pathway in bile acid biosynthesis.

It has been reported that p38 activity is needed for an active bile acid export pump (30). A potential alternative explanation for the strong reduction in 7*α*-hydoxylase expression that we observed when p38 kinase activity is suppressed either with chemical inhibitors (Fig. 1*A*) or by the use of a dominant negative p38 kinase mutant (Fig. 1*B*) could be that the bile acid export pump is blocked, which would impair bile acid export from the hepatocytes. This would result in bile acid accumulation inside the hepatocytes, suppressing 7*α*-hydroxylase transcription. In other words, our observation would be the result of an indirect effect on 7*α*-hydoxylase expression rather than direct. However, quantification of bile acid synthesis indicates that the effect of p38 kinase inhibitors is direct and not indirect, because bile acid levels are reduced between 30 and 46% outside the cells (bile acids are rapidly released in the medium) (Fig. 2). Although the decrease in bile acids inside the cells is not statistically significant, they do not increase either, further supporting the idea that the p38 kinase pathway has a direct effect on their synthesis. It should be noted that in primary hepatocytes ~50% of bile acid synthesis takes place through the acidic pathway in which 7*α*-hydroxylase is not involved, thus the highest reduction in bile acid synthesis that can be seen suppressing 7*α*-hydroxylase is 50% (31). The dramatic suppression of 7*α*-hydroxylase expression that we observed (Fig. 1) could, therefore, originate from an effect on either FTF or HNF-4*α*, because these two nuclear receptors are the only two transcription factors required for 7*α*-hydroxylase transcription, other than general transcription factors (1, 3). Protein and promoter studies are consistent with the idea that p38 kinase phosphorylates HNF-4*α*, and as a result, the half-life of HNF-4*α* is extended (Figs. 3–6).

Many nuclear receptors are phosphorylated, and phosphorylation affects their activity in different ways (16). There is also good evidence that HNF-4*α* is phosphorylated at multiple sites, mainly at serine residues, by several kinases (18). A recent report shows that p38 kinase phosphorylates HNF-4*α* at Ser^{158} in HepG2 cells (19), which is required for

interaction between HNF-4α and PC4, a coactivator involved in the activation of nitric-oxide synthase expression by interleukin-1β and peroxide (32). However, overexpression of HNF-4α S158A did not prevent 7α-hydroxylase transactivation in primary hepatocytes infected with an adenovirus encoding HNF-4α S158A (data not shown). This suggests that p38 kinase phosphorylates HNF-4α on at least one other residue. Studies are underway to identify the HNF-4α residue(s) that p38 kinase phosphorylates to maintain full 7α-hydroxylase expression, and they should provide a better understanding of bile acid metabolism and its regulation.

The p38 kinase pathway appears to be involved in the activation of 7α-hydroxylase expression by insulin (Figs. 7 and 8). Expression of 7α-hydroxylase has a dual response to insulin, *i.e.* with shorter incubation times, insulin activates 7α-hydroxylase expression, but with longer times, or at supraphysiological concentrations, insulin suppresses 7α-hydroxylase expression (Fig. 7*A*) (28, 33). The bile acid pool increases in diabetes both in humans (34) and in other mammals (35). After a meal, circulating insulin transiently increases to activate glucose metabolism. It is becoming evident that there is a concomitant increase in 7α-hydroxylase expression leading to an increase in bile acid synthesis to facilitate fat absorption in the intestine (28). The observation that insulin-induced p38 kinase increases HNF-4α protein and phosphorylation, which results in higher 7α-hydroxylase mRNA levels with no change in HNF-4α mRNA (Figs. 7 and 8), suggests a rapid mechanism for insulin-mediated induction of bile acid synthesis.

In summary, the present studies identify HNF-4α as a target of the p38 kinase and a requirement for maximum 7α-hydroxylase expression. However, we have not excluded other potential targets that could also affect 7α-hydroxylase expression, such as the peroxisome proliferator-activated receptor γ coactivator 1 (PGC-1α), a coactivator of HNF-4α and perhapsα FTF (22). It has been shown that PGC-1α is activated by p38 kinase (36) and that PGC-1α activates 7α-hydroxylase expression (22); thus, p38 kinase might also activate 7α-hydroxylase expression through PGC-1α, but our studies show that the p38 pathway is directly involved in the insulin-mediated activation of 7α-hydroxylase expression through phosphorylation of the nuclear receptor HNF-4α.

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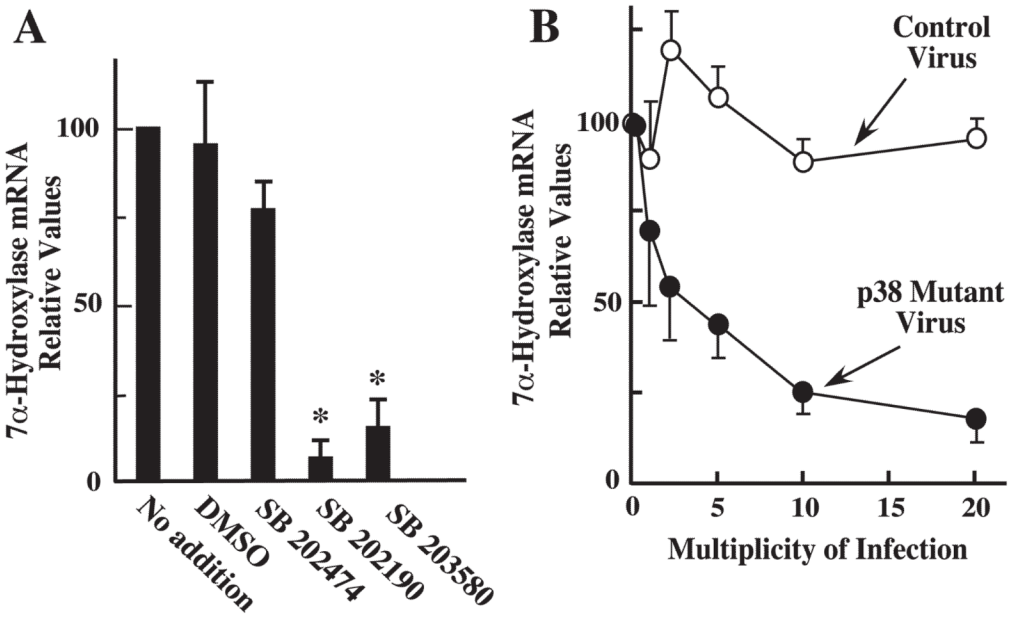

FIGURE 1. Inhibition of p38 kinase activity suppresses 7*α*-hydroxylase expression in primary rat hepatocytes

Primary rat hepatocytes were treated with the indicated chemicals (*A*) or infected with an adenovirus encoding a p38 kinase dominant negative mutant, or a control virus (*B*), as explained under "Experimental Procedures." p38 chemical inhibitors SB 202190 and SB 203580, as well as the inactive chemical analogue SB 202474, were used at 5 μM. After overnight incubation, the cells were harvested, and total RNA was extracted. 7*α*-Hydroxylase mRNA was quantified by quantitative reverse transcription PCR. Actin RNA was used to normalize 7*α*-hydroxylase mRNA values. The values represent the means ± S.D. of three experiments. In *A*, *, $p \leq 0.01$ *versus* no addition, Me_2SO (*DMSO*) and SB 202474 controls. In *B*, $p \leq 0.05$ between two groups analyzed by one-way analysis of variance.

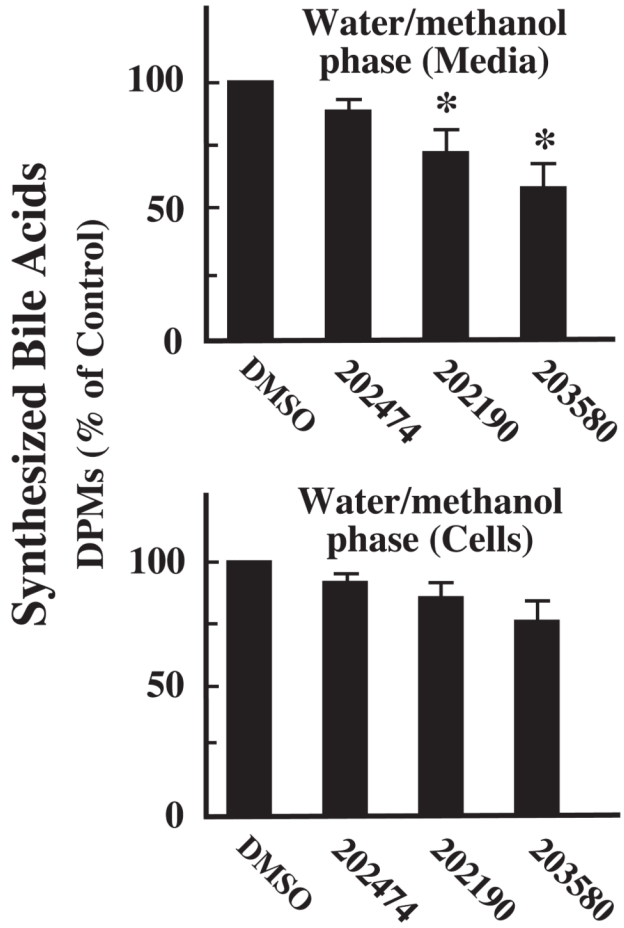

FIGURE 2. p38 kinase chemical inhibitors diminish bile acid synthesis in primary rat hepatocytes

The cells were treated as indicated and incubated with [^{14}C]cholesterol as described under "Experimental Procedures." Both medium (*top panel*) and cells (*bottom panel*) were harvested, and bile acids were extracted and quantified as described under "Experimental Procedures." Bile acid synthesis levels correspond to 100 fmol/min/mg cellular protein in the control medium and of 30 fmol/min/mg protein in the cells. They are expressed as percentages of untreated controls. The values represent the means ± S.D. of three experiments. *, $p \leq 0.05$ *versus* Me_2SO (*DMSO*) and SB 202474 controls. *DPMs*, disintegrations per minute.

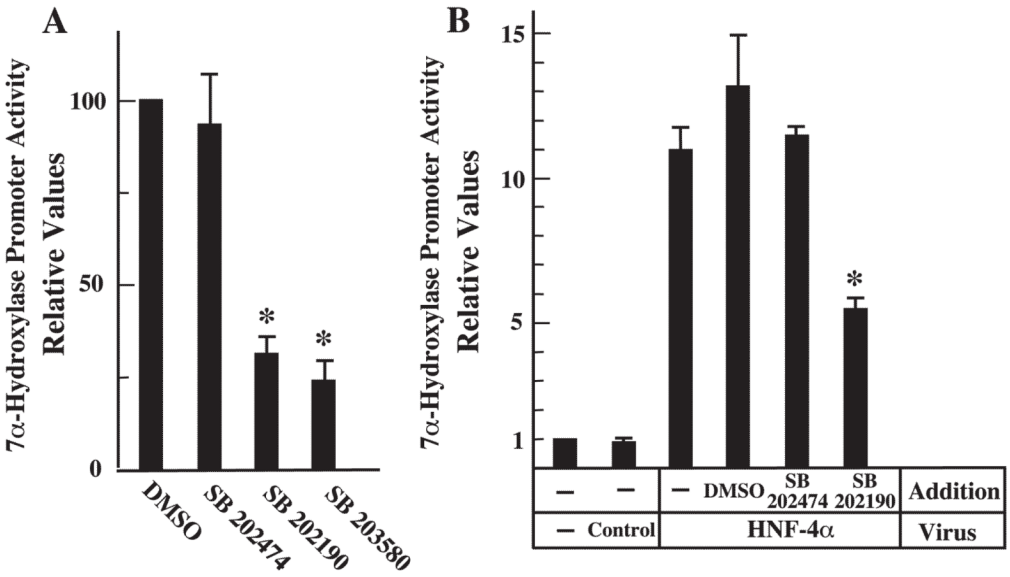

FIGURE 3. p38 kinase inhibitors suppress 7*α*-hydroxylase promoter activity through HNF-4*α*

A, wild-type 7*α*-hydroxylase promoter reporter plasmid (pGL3-R7*α*-342) was transfected into primary rat hepatocytes as described under "Experimental Procedures." After transfection the cells were treated as indicated. Forty hours later, the cells were harvested, and the luciferase and galactosidase activities were quantified. Promoter activities were calculated as the ratios of luciferase/galactosidase and expressed as the percentages of Me_2SO-treated (*DMSO*) controls. The values represent the means ± S.D. of three experiments. *, $p \leq 0.05$ *versus* Me_2SO and SB 202474 controls. *B*, CV-1 cells were infected with the indicated virus and then transfected with the wild-type 7*α*-hydroxylase promoter reporter plasmid as described under "Experimental Procedures." After transfection the cells were treated with chemical inhibitor as indicated. Promoter activities were calculated as above and expressed as relative numbers to the noninfected cells. The values represent the means ± S.D. of three experiments. *, $p \leq 0.01$ *versus* no addition, Me_2SO and SB 202474 controls.

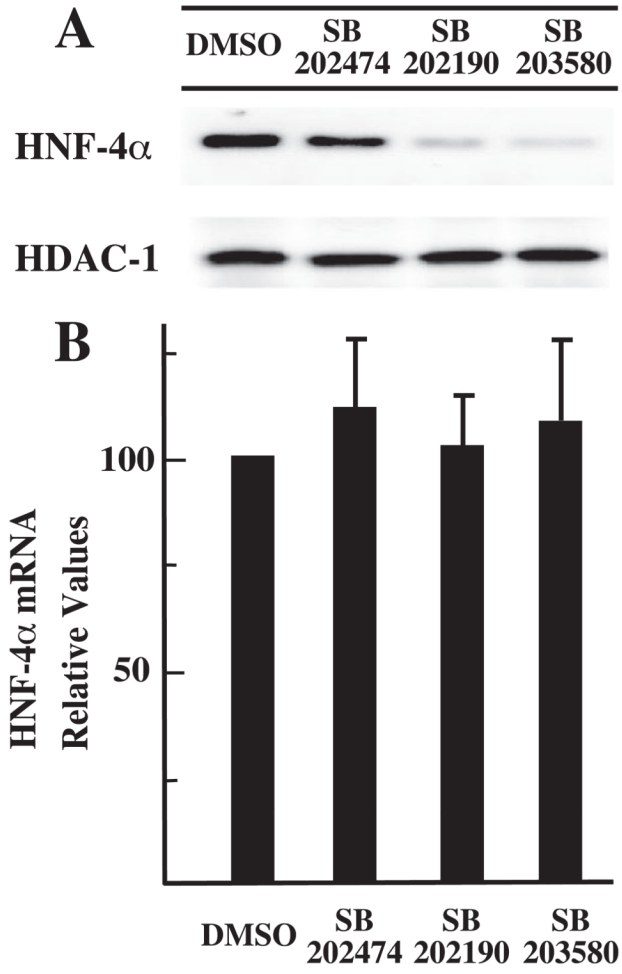

FIGURE 4. p38 chemical inhibitors suppress HNF-4*α* protein, but not mRNA, in primary rat hepatocytes

Primary rat hepatocytes were treated as in the experiments described in the legend to Fig. 1 with the indicated chemicals as in Fig. 1. *A*, 20 h after treatment, nuclear extracts were prepared and used in Western blotting analysis to quantify HNF-4*α*. Histone deacetylase 1 was also quantified as control. A representative experiment is shown. *B*, another set of equally treated plates were used to prepare total RNA and used to quantify HNF-4*α* mRNA by Q-PCR. The values represent the means ± S.D. of three experiments. There were no significant differences in all groups. *DMSO*, Me_2SO.

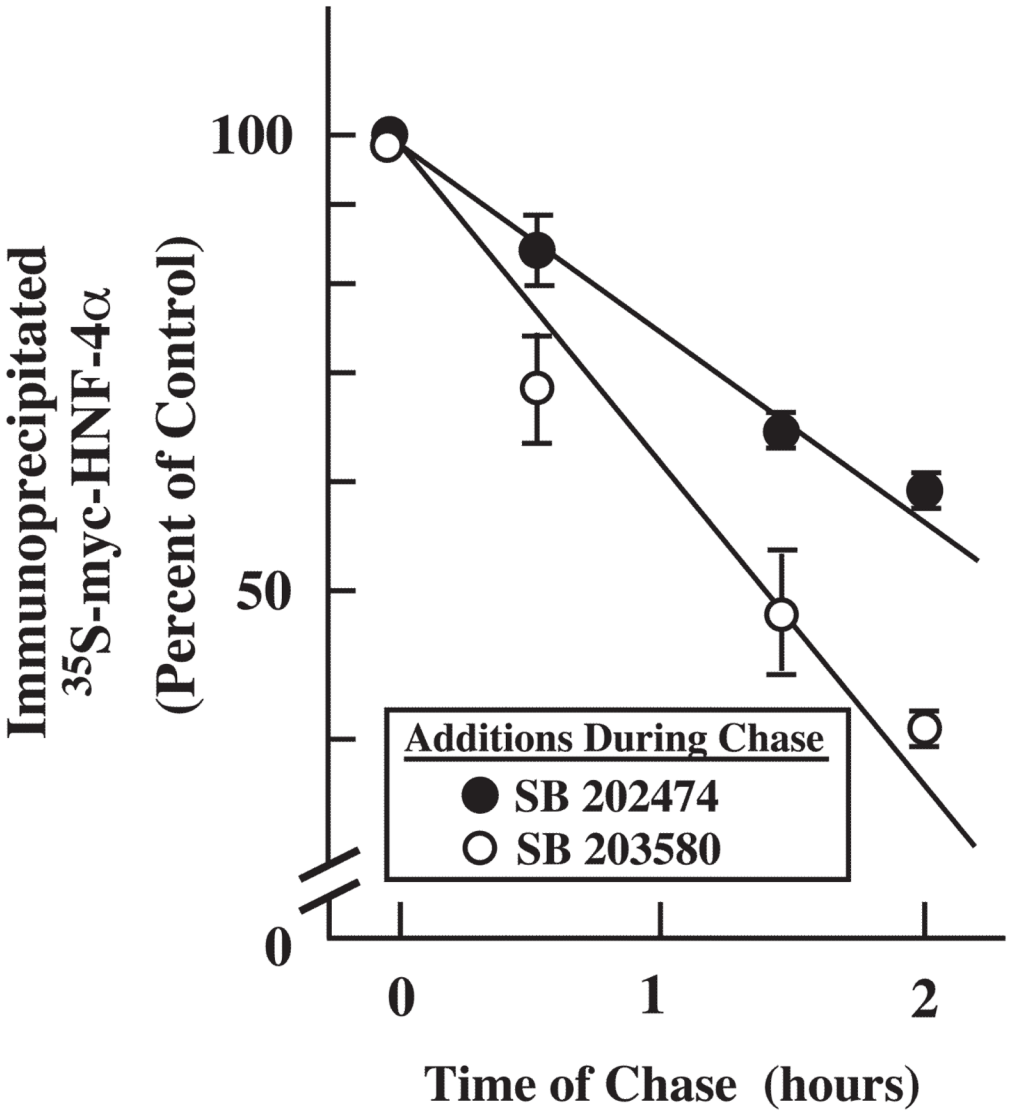

FIGURE 5. p38 kinase inhibitor accelerates HNF4*α* degradation in primary rat hepatocytes

Pulse-chase experiments were performed in primary rat hepatocytes infected with an adenovirus carrying a cytomegalovirus-HNF-4*α* chimeric gene to overexpress HNF-4*α*. After metabolic labeling as described under "Experimental Procedures," the cells were incubated with 5 μM p38 inhibitor SB 203580 and inactive control SB 202474 and then harvested at the indicated times. Labeled HNF-4*α* was immunoprecipitated with anti-Myc antibodies and quantified by scintillation counting. The values represent the means ± S.D. of five experiments. For the 0.5-h point, $p \leq 0.005$ *versus* their corresponding SB 202474 control. For the 1-h time point, $p \leq 0.01$ *versus* its corresponding SB 202474 control. For the 1.5-h time point, $p \leq 0.001$ *versus* its corresponding SB 202474 control.

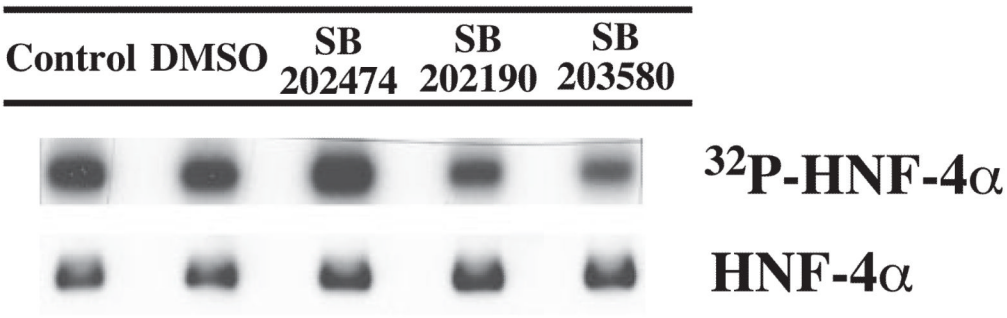

FIGURE 6. HNF-4*α* phosphorylation in primary rat hepatocytes is partially blocked by p38 kinase inhibitors

Rat primary hepatocytes were infected with Myc-HNF4*α* adenovirus overnight and metabolically labeled as indicated under "Experimental Procedures" in the presence of the p38 chemical inhibitors SB 202190 and SB 203580, the inactive chemical analogue SB 202474, or the vehicle Me_2SO for as indicated for 2 h. The cell extracts were immunoprecipitated with an anti-Myc antibody, then loaded on SDS-PAGE, and subjected either to autoradiography or Western blotting using an anti-HNF-4*α* antibody. *DMSO*, Me_2SO.

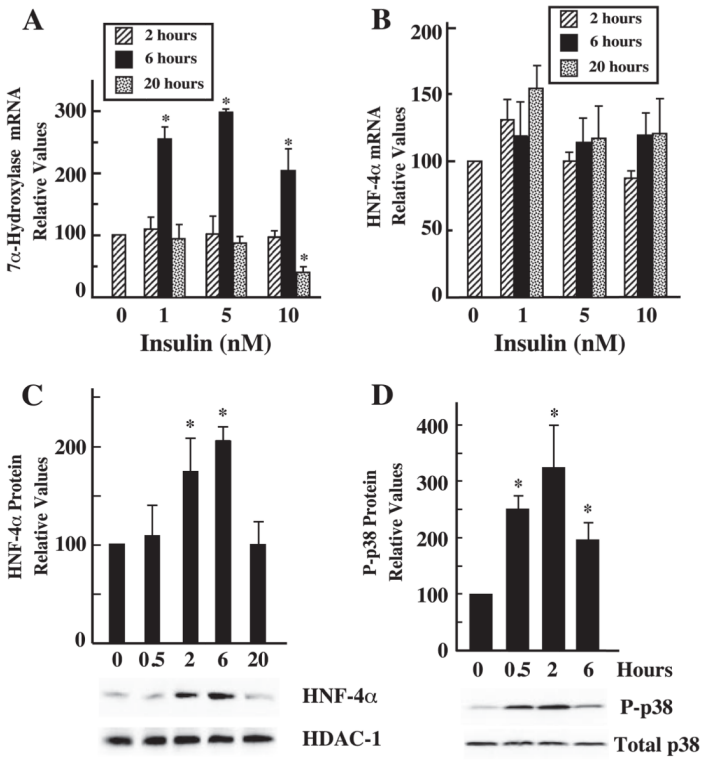

FIGURE 7. Concomitant activation of 7*α*-hydroxylase expression, p38 kinase, and HNF-4*α* protein

Primary rat hepatocytes were treated with the indicated insulin concentrations for 2, 6, or 20 h and harvested, and 7*α*-hydroxylase mRNA (*A*) and HNF-4*α* mRNA (*B*) were quantified. *C*, rat hepatocytes were incubated with 10 nM insulin for the indicated times. The cells were harvested, and nuclear extracts were prepared. HNF-4*α* and histone deacetylase 1 as a control were quantified in the nuclear extracts. *D*, phospho-p38 kinase (*P-p38*) and total p38 as a control were quantified by Western blotting in the cytoplasmic extracts of primary hepatocytes incubated with 10 nM insulin for the indicated times. The values represent the means ± S.D. of three experiments. *, $p \leq 0.05$ *versus* the non-insulin control. In *C* and *D*, a representative experiment is shown at the *bottom*.

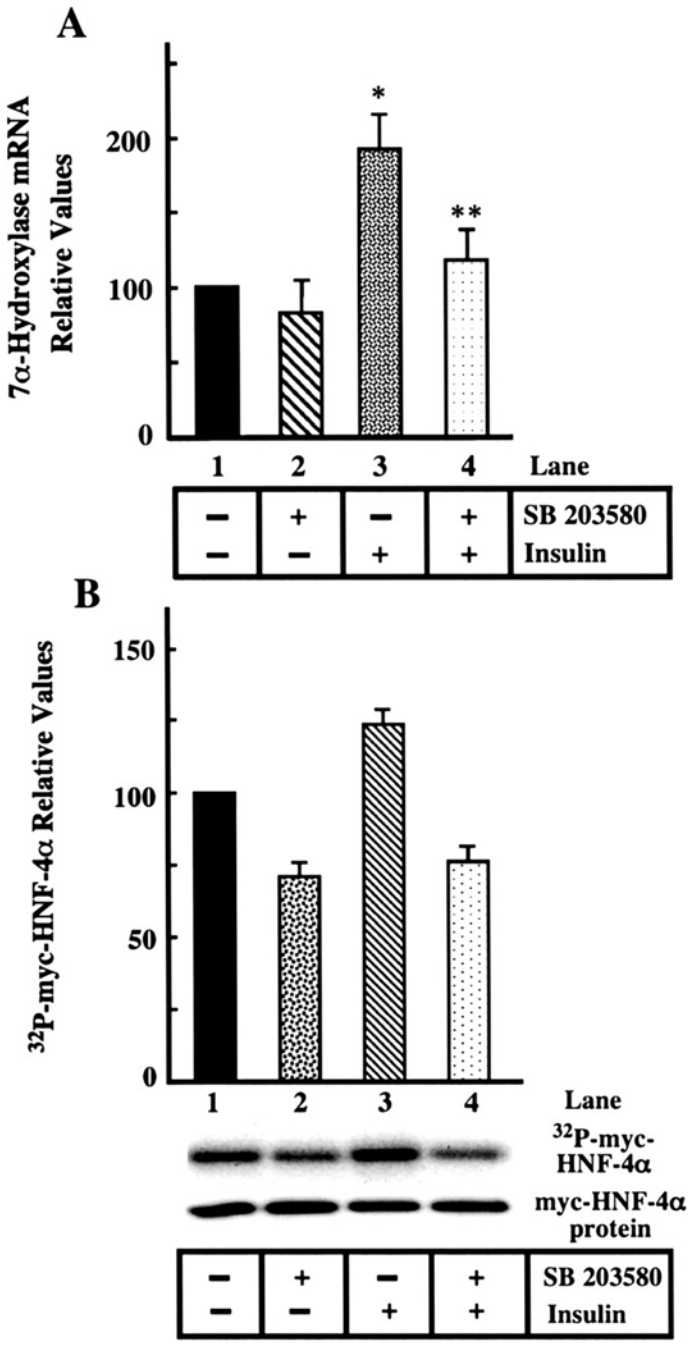

FIGURE 8. Mitogen-activated protein p38 kinase inhibitor prevents insulin-mediated activation of HNF-4*α* expression and phosphorylation

A, primary rat hepatocytes were incubated with either the p38 kinase inhibitor SB 203580 (5 μM) or vehicle (Me_2SO) for 30 m. Then insulin was added to a final concentration of 10 nM where indicated for 6 h. The cells were harvested, total RNA was prepared, and 7α-hydroxylase mRNA was quantified as indicated under "Experimental Procedures." The values represent the means ± S.D. of four experiments. *, $p \leq 0.01$ *versus* the no insulin/no SB (*lane 1*). **, $p \leq 0.01$ *versus* the insulin/no SB (*lane 3*). There were no statistically significant differences between *lanes 3* and *4*. In *B*, the cells were infected with an adenovirus containing a Myc-HNF-4α fusion cDNA. After overnight expression, the cells

were incubated in phosphate-free medium in the presence of the p38 kinase inhibitor SB 203580, where indicated, for 2 h. Then insulin was added where indicated to a final concentration of 10 nM, and after 1 h, 0.5 mCi of [^{32}P]phosphate was added. The cells were harvested 1 h later, Myc-HNF-4*α* immunoprecipitated, separated by PAGE, and visualized by autoradiography, and the bands were sliced and quantified. The values represent the means ± S.D. of three experiments. $p \leq 0.05$ between *lanes 1* and *2* and *lanes 1* and *3*. $p \leq 0.01$ between *lanes 3* and *4*. Another aliquot of the ^{32}P-labeled samples was subjected to PAGE, transferred to nitrocellulose, and developed for Myc-HNF-4*α* protein levels by Western blotting. A representative experiment is shown at the *bottom*.

TABLE 1

Oligonucleotides used in the quantitative reverse transcription PCR quantification of RNAs

For the quantitative reverse transcription PCR oligonucleotides, the nucleotide numbers correspond to the GenBank™ numbers. An F in the oligonucleotide name means forward, an R means reverse, and a T means TaqMan probe.

Oligonucleotide	Gene	GenBank™ accession number	Nucleotides	Sequence
r7*α*-F	Rat CYP7A1	NM_012942	989–1010	CAT CTC AAG CAA ACA CCA TTC C
r7*α*-R			977–1001	AGC ACT CTG TAA AGC TCC ACT CAC T
r7*α*-T			931–960	TCA TTG CTT CAG GAC TCC TGA TCA TTT GAA
rHNF4-F	Rat HNF-4*α*	NM_022180	897–916	CAA GGC TGT GGG CTC CAT AG
rHNF4-R			972–992	ATT GTT GGT CAC CTC CGT GTC
r*β*-Actin-F	Rat *β*–Actin	NM_031144	574–594	TCT ACG AGG GCT ATG CTC TCC
r*β*-Actin-R			699–721	TCT TTG ATG TCA CGC ACG ATT TC
r*β*-Actin-T			605–626	CCT GCG TCT GGA CCT GGC TGG C