Tauro lithocholic Acid-3 Sulfate Impairs Insulin Signaling in Cultured Rat Hepatocytes and Perfused Rat Liver

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Key Words

Bile acids • PKB • Proteolysis • Rapamycin • Tauroursodeoxycholate • Insulin Resistance • Cell volume

Abstract

Background/Aims: The role of bile acids for insulin resistance in cholestatic liver disease is unknown. **Methods:** The effect of tauro lithocholic acid-3 sulfate (TLCS) on insulin signaling was studied in cultured rat hepatocytes and perfused rat liver. **Results:** TLCS induced insulin resistance at the level of insulin receptor (IR) β Tyr(1158) phosphorylation, phosphoinositide (PI) 3-kinase activity and protein kinase (PKB) Ser(473) phosphorylation in cultured hepatocytes. Consistently, the insulin stimulation of the PI 3-kinase-dependent K⁺ uptake, hepatocyte swelling and proteolysis inhibition was blunted by TLCS in perfused rat liver. The PKC inhibitor Gö6850 and tauroursodeoxycholate (TUDC) counteracted the suppression of insulin-induced IR β and PKB phosphorylation by TLCS. Rapamycin and dibutyryl-cAMP, which inhibited basal signaling via mammalian target of rapamycin (mTOR), restored insulin-induced PKB- but not IR β phosphorylation. In livers from 7 day bile duct-ligated rats PKB Ser(473) phosphorylation was

decreased by about 50%. **Conclusion:** TLCS induces insulin resistance by a PKC-dependent suppression of insulin-induced IR β phosphorylation and the PI 3-kinase/PKB path. This can in part be compensated by a decrease of mTOR activity, which may release insulin-sensitive components downstream of the insulin receptor from tonic inhibition. The data suggest that retention of hydrophobic bile acids confers insulin resistance on the cholestatic liver.

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Introduction

Bile acids are increasingly considered as regulators of protein-, lipid- and carbohydrate metabolism [1]. In cholestasis, hepatocellular retention of hydrophobic bile acids triggers liver injury, which can be antagonized by the hydrophilic (tauro)ursodeoxycholate [(T)UDC] (for review see [2, 3]).

Bile acids activate a variety of distinct signaling pathways. In hepatocytes, individual bile acids activate integrins [4], the insulin receptor (IR) [5], the epidermal growth factor (EGF) receptor [6, 7] or the CD95 death receptor [6, 8] in a ligand-independent manner. The nuclear bile acid receptor farnesoid-X-receptor (FXR) α

among others takes part in the genomic control of hepatic bile acid synthesis and transport [9].

Progress has been achieved in linking non-genomic bile acid signaling to alterations of hepatic metabolism, transport and viability. For example, the choleretic effect of TUDC in perfused rat liver was attributed to the integrin- and Src-dependent activation of Erk- and p38-type MAP-kinases, resulting in a rapid insertion of the bile salt export pump (Bsep) into the canalicular membrane [4]. Stimulation of glycogen synthesis by deoxycholate (DCA) is due to a PI 3-kinase-dependent PKB activation, which was mapped downstream of a DCA-induced IR β activation [5]. Interestingly, IR β inhibition increased DCA toxicity [10], whereas in the case of tauroolithocholic acid 3-sulfate (TLCS) and glycochenodeoxycholate (GCDC) inhibition of the EGF receptor protected hepatocytes from CD95 activation and execution of apoptosis [7].

In the liver a cross talk between bile acid-induced signaling and pathways triggered by glucagon or cytokines has been established. For example, TUDC in hamster hepatocytes inhibits cAMP synthesis by glucagon [11] and UDC inhibits the interleukin (IL)-6 induction of phospholipase (PL)A₂Ila expression at the transcriptional level in HepG2 cells [12]. As shown recently, in perfused rat liver, rat hepatocytes and HepG2 cells GCDC stimulates a caspase-dependent cleavage of gp130, which was prevented by TUDC [13]. Furthermore, a p38^{MAPK}-dependent inhibition of IL-6-induced STAT3 tyrosine phosphorylation with impact on the acute phase response and liver regeneration was observed [13]. Also the interferon γ -induced expression of the antiviral proteins 2',5' oligoadenylate synthetase and MxA was antagonized by bile acids [14].

There is indirect evidence that specific bile acids might interfere with PI 3-kinase-dependent signaling in the liver. In rat hepatocytes cultured in the presence of serum both, TUDC and TLCS rapidly stimulated a PI 3-kinase-dependent PKB Ser(473) phosphorylation, which in the case of TLCS was followed by a sustained suppression of PKB phosphorylation below control levels [15, 16]. In the present study, the effect of TLCS on hepatic insulin signaling was studied in cultured rat hepatocytes and perfused rat liver. The data show that TLCS induces insulin resistance in cultured rat hepatocytes by a PKC-dependent impairment of insulin-induced IR β Tyr(1158) and PKB Ser(473) phosphorylation that was antagonized by TUDC. Consistently, insulin stimulation of the PI 3-kinase-dependent K⁺ uptake, cell swelling and proteolysis inhibition was blunted by TLCS in perfused rat liver

and livers of 7 days bile-duct ligated rats displayed decreased levels of PKB (Ser473) phosphorylation. It is suggested that hydrophobic bile acids confer insulin resistance on the cholestatic liver.

Materials and Methods

Materials

Antibodies recognizing Erk-1/Erk-2, IR β , Tyr(1158)-phosphorylated IR β , IRS1, and Ser(473)-phosphorylated PKB were from Upstate (Charlottesville, VA, USA); antibodies against p70 S6 kinase and PKB were from Transduction Laboratories (Heidelberg, Germany). Antibodies against Thr(421)/Ser(424)-phosphorylated p70 S6 kinase and 4E-BP1 were from Cell Signaling (Frankfurt am Main, Germany); the antibody against phospho-p38^{MAPK} was from Promega (Madison, USA). PD098059, SB203580, wortmannin, LY294002, rapamycin, AG1478 and apocynin were from Calbiochem-Novabiochem GmbH (Darmstadt, Germany). Gö6850 was a gift from Gödecke AG (Freiburg, Germany). Cell culture media and fetal calf serum were from Gibco Life Technologies (Gaithersburg, MD). The antibody against HSC70, EDTA, sodium vanadate, insulin, NP-40, Triton X-100, AY9944, epigallocatechin gallate, taurocholate, tauroursodesoxycholate and tauroolithocholic acid-3 sulfate were from Sigma (Munich, Germany). The PKC ζ -inhibitory pseudosubstrate myristoyl-Ser-Ile-Tyr-Arg-Arg-Gly-Ala-Arg-Arg-Trp-Arg-Lys-Leu-OH was synthesized by Dr. Ralf Hoffmann (Biologisch-Medizinisches Forschungszentrum, Heinrich-Heine-University, Düsseldorf, Germany). L-[4,5-³H] leucine, [³H]inulin and [¹⁴C]urea were from Amersham (Braunschweig, Germany). L-lactic acid was from Roth (Karlsruhe, Germany). All other chemicals were from Merck (Darmstadt, Germany).

Hepatocyte preparation and cell culture

Isolated hepatocytes were prepared from livers of male Wistar rats, fed ad libitum with a standard diet by a collagenase perfusion technique. Aliquots of 1.5×10^6 cells were plated on collagen-coated on Cluster six dishes (Falcon, Heidelberg, Germany) and maintained at 37°C at a 5% CO₂ atmosphere in bicarbonate-buffered Krebs-Henseleit medium (115 mmol/l NaCl / 25 mmol/l NaHCO₃ / 5.9 mmol/l KCl / 1.18 mmol/l MgCl₂ / 1.23 mmol/l NaH₂PO₄ / 1.2 mmol/l Na₂SO₄ / 1.25 mmol/l CaCl₂), supplemented with 6 mmol/L glucose. After 2 hours, the culture was continued for 24 h in 1.5 ml DMEM medium supplemented with penicillin (100 U/ml), streptomycin (0.1 mg/ml) and 10% FCS. Then the medium was removed and hepatocytes were cultured for another 6 h in serum-free medium of otherwise identical composition or containing additions for experimental treatment as indicated. H4IIE-C3 rat hepatoma cells (A.T.C.C. CRL 1600) were cultured on Cluster six dishes in DMEM-F12 / 5% CO₂ / 5mM glucose at 37°C, pH 7.4, supplemented with 10% FCS. When cells had reached 90% confluency, they were washed with Dulbeccos PBS and cultured for another 6 h in serum-free medium of otherwise identical composition or containing additions for experimental treatment as indicated.

Western Blot analysis

Medium was removed from the culture and cells were immediately lysed at 4°C using 10 mM Tris/HCl buffer (pH 7.4) containing 1% Triton X-100, 0.5% NP-40, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 20 mM NaF and 1 tablet/50 ml of protease inhibitor cocktail (Roche Diagnostics GmbH, Mannheim, Germany). The homogenized lysates were centrifuged at 20,000 g at 4°C for 10 min. SDS polyacrylamide gel electrophoresis and western blot analysis was performed according to standard procedures as described [17]. Blots were blocked overnight in 1–5% bovine serum albumin solubilized in 20 mM Tris/HCl (pH 7.5) containing 150 mM NaCl and 0.1% Tween 20 and then incubated for 3–4 h with the respective antibody diluted 1:1,000–1:20,000. Following washing and incubation for 2 h with horseradish peroxidase-coupled anti-rabbit-IgG antibody or horseradish peroxidase-coupled anti-mouse-IgG antibody (1:10,000–1:20,000, 4°C) the blots were washed again and developed using enhanced chemiluminescent detection (Amersham Biosciences Europe, Freiburg, Germany). Densitometric analysis was performed with the E.A.S.Y RH system (Herolab, Wiesloch, Germany) and the Scion Image software (Scion Corporation, Frederick, MD, USA).

PI 3-kinase assay

Aliquots containing 100 µg protein were incubated with 1.5 µg of the polyclonal antibody against IRS1 for 2 hours at 4°C. Immune complexes were collected by using protein A-Sepharose 4B. PI 3-kinase activity was assayed as described previously [15].

Liver Perfusions

The experiments were approved by the responsible local authorities. Livers from male Wistar rats (120 – 150 g body wt), fed a standard chow, were perfused in a nonrecirculating manner according to the published standardized protocol ([18] and references therein). The effluent K⁺ concentration was continuously monitored with a K⁺-sensitive electrode (Radiometer, Munich, Germany); insulin-induced K⁺ fluxes were determined by planimetry of areas under curves [18].

The rate of proteolysis was assessed by measuring the release of [³H] from isolated perfused rat livers after prelabeling of liver proteins in vivo by intraperitoneal injection of 150 µCi of L-[4,5-³H]leucine 16 – 20 hours before the perfusion experiment as described [18]. In order to correct for different labeling of hepatic proteins after intraperitoneal injection in the individual perfusion experiment, the rate of proteolysis was set to 100% under control conditions and the extent of proteolysis inhibition was determined 30 min after institution of the respective condition, a time point when a new steady state had been reached.

The intracellular water space in the intact perfused rat liver was calculated from the difference of washout profiles of simultaneously infused [¹⁴C]urea and [³H]inulin (approximately 500 Bq/ml perfusate), as described [18].

Bile duct ligation of rats

Male Sprague Dawley rats (weighing 200 – 250 g) were kept with free access to stock diet and water. The animals

received care according to the criteria outlined in the “Guide for the Care and Use of Laboratory Animals” (NIH publication, 86-23, revised 1985). The study protocol was approved by the Animal Welfare Committee at the Regierungspräsidium Düsseldorf. Bile duct ligation (BDL) and preparation of liver samples 1, 3 or 7 days after BDL was performed as recently described [19].

Analysis of results

Results from independent experiments are expressed as means ± SEM. Results were compared using the Student's t-test: $p \leq 0.05$ was considered statistically significant.

Results

Effect of bile acids on insulin-induced PKB phosphorylation in cultured rat hepatocytes and H4IIE rat hepatoma cells

The effect of bile acids on insulin-induced PKB phosphorylation was studied in one day-cultured rat hepatocytes. Hepatocytes were pre-treated with 100 µmol/l taurocholate (TC), tauroolithocholic acid 3-sulfate (TLCS), glycochenodeoxycholate (GCDC), tauroursodeoxycholate (TUDC) for 6 h in the absence of serum. Thereafter, the hepatocytes were stimulated with 100 nmol/l insulin for another 5 min. Figure 1 shows that insulin potently increased PKB Ser(473) phosphorylation, which was not affected by TC or TUDC (lines 1, 2, 5, and 7). On the other hand, both, TLCS and GCDC decreased PKB phosphorylation in response to insulin by about 50 % (lines 1, 3, 4). Bile acids were without effect on basal PKB phosphorylation in the absence of insulin (lines 7–11). TLCS and GCDC significantly decreased overall PKB expression to 52.8 ± 9.8 and $61.3 \pm 9.3\%$ respectively ($n=3$). Levels of the constitutively expressed heat shock protein HSC70 remained stable in presence of TLCS, but were decreased by GCDC (lines 4 and 10). Preincubation of the hepatocytes with taurine (24 h, 10 mmol/l), a compatible organic osmolyte accumulated by hepatocytes [20], was without effect on insulin-induced or basal PKB Ser(473) phosphorylation (Figure 1, lines 6, 12).

As shown in Figure 2, TLCS already at a concentration of 10 µmol/l impaired PKB phosphorylation by insulin.

Bile acid effects on signal transduction and gene expression in hepatocytes critically depend on cellular bile acid uptake [16, 21, 22]. The hepatocytes' capacity to accumulate bile acids disappears in the course of culture due to downregulated expression of the Na⁺-taurocholate cotransporting polypeptide [23]. Also in H4IIE rat

Fig. 1. Effect of bile acids and taurine on PKB phosphorylation by insulin and HSC70 expression in cultured rat hepatocytes: Hepatocytes were serum-starved and exposed for 6 h to 100 $\mu\text{mol/l}$ taurocholate (TC), tauroolithocholylsulfate (TLCS), glycochenodeoxycholate (GCDC), tauroursodeoxycholate (TUDC) or 10 mmol/l taurine, which in order to allow accumulation inside the hepatocytes was already present 24 h before the change to serum free medium. Thereafter, hepatocytes were stimulated with 100 nmol/l insulin (lines 1-6) or remained without insulin addition (lines 7-12) for another 5 min. Proteins were analyzed by Western blot for the presence of Ser(473)-phosphorylated PKB, overall PKB and HSC70, respectively. Changes of PKB phosphorylation and HSC70 expression levels were monitored by densitometry. PKB Ser(473) (pPKB) expression was normalized to overall PKB expression. PKB phosphorylation and HSC70 expression in absence of bile acids, taurine and insulin (line 7) was set to 1. *, PKB phosphorylation by insulin was significantly ($p \leq 0.05$) decreased compared to the control stimulation by insulin (line 1). §, HSC70 expression significantly ($p \leq 0.05$) decreased compared to that found in the absence of bile acids, taurine and insulin (line 7). The figure summarizes data from 3 independent experiments.

hepatoma cells concentrative bile acid uptake is markedly downregulated [24]. Figure 3 shows that PKB phosphorylation was insensitive to TLCS and GCDC in 2 day-cultured hepatocytes (Figure 3A) and H4IIE cells (Figure 3B) stimulated with insulin (lines 1-6, 7-12). Further, overall PKB expression was not affected by the bile acids in these cells. The findings suggest that

Fig. 2. TLCS-induced suppression of PKB phosphorylation by insulin in cultured rat hepatocytes: dependence on TLCS concentration: Hepatocytes were pretreated for 6 h with 100, 50 or 10 $\mu\text{mol/l}$ TLCS (line 2-4) or remained without TLCS addition (lines 1, 5 and 6). Thereafter, hepatocytes were stimulated (lines 1-5) or not (line 6) for 5 min with 100 nmol/l insulin. Changes of PKB Ser(473) phosphorylation were monitored by densitometry and normalized to overall PKB expression. PKB phosphorylation in insulin-stimulated cells (TLCS absent) was set to 1. *, PKB phosphorylation significantly ($p \leq 0.05$) different from that in cells stimulated with insulin in absence of TLCS. (n=3).

the uptake of TLCS and GCDC is important for modulation of insulin signaling by these compounds.

In order to examine whether TLCS and GCDC affect PKB phosphorylation following a pre-stimulation of hepatocytes with insulin, cells were exposed to insulin for 10 min. Then 100 $\mu\text{mol/l}$ TLCS or GCDC were added and PKB phosphorylation after another 10, 30, 60 and 120 min was examined. Figure 4 shows that TLCS or GCDC after 10 min significantly added to PKB phosphorylation in the presence of insulin. In absence of the bile acids insulin further increased PKB phosphorylation at the 60 and 120 min time points (Figure 4, lines 3, 9 and 12), which, however, was not the case in presence of GCDC (Figure 4, lines 10 and 13) and TLCS (Figure 4, lanes 11 and 14), respectively. Further, at the 120 min time point PKB Ser(473) phosphorylation by insulin was decreased in the presence of TLCS compared to that found in absence of the bile acid at this time point (Figure 4, lines 12 and 14).

Fig. 3. Effect of TLCS and GCDC on PKB phosphorylation by insulin in 2 day-cultured rat hepatocytes and H4IIE rat hepatoma cells: 2 day-cultured hepatocytes (A) or H4IIE rat hepatoma cells (B) were pretreated with 100 μ mol/l TLCS or GCDC for 6 h and then exposed to insulin (100 nmol/l) for another 5 min as indicated. PKB Ser(473) phosphorylation changes were monitored as described in the legend to Figure 2. *, PKB phosphorylation significantly ($p \leq 0.05$) different from that in cells stimulated with insulin in absence of bile acids. ($n=3$).

Effect of TLCS on PI 3-kinase activation by insulin

PKB phosphorylation by insulin is a direct consequence of PI 3-kinase activation [25]. Insulin stimulates the recruitment of the PI 3-kinase adaptor subunit p85 by tyrosine-phosphorylated insulin receptor substrates including IRS1, which leads to activation of the catalytic p110 subunit [25]. As shown in Figure 5, TLCS decreased the PI 3-kinase activity in insulin-stimulated hepatocytes which was coimmunoprecipitated by an anti-IRS1 antibody. Consistent with earlier observations [16] TLCS was without effect on basal PI 3-kinase activity in the absence of insulin.

Pharmacological characterization of the suppression of insulin-induced PKB phosphorylation by TLCS

TLCS activates the extracellular signal-regulated MAP-kinase Erk-2, the c-Jun-N-terminal kinase JNK1 and the $p38^{\text{MAPK}}$ [16]. Hepatocytes were exposed to TLCS in the presence of specific MAP-kinase pathway inhibitors in order to examine the involvement of MAP-kinases in the suppression of insulin-induced PKB phosphorylation by TLCS (Figure 6). PD98059, which blocks the Erk pathway at the level of MAP-kinase kinases [26], was without effect on the suppression of PKB phosphorylation by TLCS (Figure 6A, lines 1-6) but was able to decrease Erk-1/Erk-2 phosphorylation in the presence of insulin plus TLCS (Figure 6B, line 1-3). Similarly, the JNK inhibitor SP600125 [27] did not affect the suppression of insulin-induced PKB phosphorylation by TLCS (Figure 6A, lines 7-12) but effectively decreased JNK1 phos-

phorylation observed in the presence of TLCS (Figure 6B, lines 4-6). It was suggested, that an inhibition of JNK phosphorylation by SP600125 relates to inhibition of the JNK kinase MKK4 and/or JNK auto-phosphorylation by the compound [27]. SB203580, a specific $p38^{\text{MAPK}}$ inhibitor [28], further decreased PKB phosphorylation in presence of TLCS plus insulin (lines 13-18). As expected, SB203580 prevented the phosphorylation of the $p38^{\text{MAPK}}$ substrate MAP-kinase-activated protein kinase (MAPKAP)2 (Figure 6B, lines 7-12). Thus, $p38^{\text{MAPK}}$ activity may counteract the suppression of PKB phosphorylation by TLCS.

The impact of protein tyrosine phosphatases on the TLCS-induced suppression of PKB phosphorylation in insulin-stimulated hepatocytes was addressed by the use of the Ser/Thr phosphatase inhibitor okadaic acid (OA) and the tyrosine phosphatase inhibitor vanadate (Van). Both compounds had no influence on PKB phosphorylation in the presence of TLCS plus insulin (Figure 7, lines 1-12).

Caspase inhibition and dibutyl-cAMP were shown to prevent TLCS-induced apoptosis of rat hepatocytes [16, 29]. As shown in Figure 7 (lines 13-18), the pancaspase inhibitor FMK001 was without effect on the suppression of insulin-induced PKB phosphorylation by TLCS, suggesting that caspase-dependent signaling was not involved. On the other hand, dibutyl-cAMP strongly increased the insulin-induced PKB phosphorylation in hepatocytes exposed to TLCS (lines 19-24). Dibutyl-cAMP *per se* did not increase PKB phosphorylation (line 24) but slightly increased PKB phosphorylation in the presence of TLCS (line 23).

Fig. 4. Effect of TLCS and GCDC on PKB phosphorylation in cultured rat hepatocytes pre-treated with insulin: Hepatocytes were stimulated with insulin (100 nmol/l) for 5 min. Then, 100 μ mol/l TLCS or GCDC were added where indicated and the culture was continued for another 10, 30, 60 and 120 min. Changes of PKB Ser(473) phosphorylation were monitored by densitometry and normalized to overall PKB expression. PKB phosphorylation following a 5 min exposure of the cells to insulin (bile acids absent, “zero” time point, lane 2) was set to 1 and PKB phosphorylation at the following time points is given as a fraction thereof. *, PKB phosphorylation significantly ($p \leq 0.05$) different from that found in insulin-stimulated cells at the “zero” time point; §, a significant ($p \leq 0.05$) decrease of PKB phosphorylation compared to that found in absence of bile acids at the respective time point ($n=4$).

Gö6850 inhibits a broad spectrum of PKCs including PKC α , β I, β II, γ , δ and ϵ but not ζ [30]. Figure 7 shows that Gö6850 increased the insulin-induced PKB phosphorylation in presence of TLCS (lines 25-30). Also rapamycin, a highly specific inhibitor of the mammalian target of rapamycin (mTOR) [31], increased the insulin-induced PKB phosphorylation in presence of TLCS. Both, Gö6850 and rapamycin were without effect on PKB phosphorylation in the absence of insulin. It is suggested that PKC isoforms and mTOR contribute to the suppression of PKB phosphorylation by TLCS.

TLCS-induced apoptosis of cultured rat hepatocytes depends on CD95 activation, which was mapped downstream of a sphingomyelinase- PKC ζ - and NADPH oxidase-dependent activation of the EGF receptor [7, 32]. Here, the potential involvement of this pathway in the suppression of insulin-induced PKB phosphorylation by TLCS was examined (Figure 7, lines 37-66). Neither the sphingomyelinase inhibitor AY9944, nor the myristoylated PKC ζ inhibitory pseudosubstrate peptide SIYRRGARRWRKL or the NADPH oxidase inhibitor apocynin, which effectively prevented TLCS-induced ROS production and apoptosis [32], did alter the suppression of the insulin-induced PKB phosphorylation by TLCS. (Figure 7, lines 37-54). Also the antioxidative flavonoid epigallocatechin gallate (EGCG) was without effect on the suppression of insulin-induced PKB phospho-

Fig. 5. Effect of TLCS on PI 3-kinase activation by insulin: Hepatocytes were pretreated or not for 6 h with 100 μ mol/l TLCS. Where indicated cells were stimulated with insulin (100 nmol/l) for another 5 min. PI 3 kinase activity precipitated with an anti-IRS1 antibody was assayed. PI 3-kinase activity in presence of insulin (without TLCS pre-treatment) was set to 1 and PI 3-kinase activity in presence of insulin plus TLCS is given as a fraction thereof. *, PI 3-kinase activity significantly ($p \leq 0.05$) different from that found following insulin stimulation in absence of TLCS. ($n=3$).

Fig. 6. Effect of MAP-kinase pathway inhibitors on the suppression of insulin-induced PKB phosphorylation by TLCS in cultured rat hepatocytes: (A) Hepatocytes were pretreated for 6 h with TLCS (100 $\mu\text{mol/l}$) in presence of PD98059 (20 $\mu\text{mol/l}$, lanes 1-6), SP600125 (SP, 50 $\mu\text{mol/l}$, lanes 7-12) or SB203580 (SB, 20 $\mu\text{mol/l}$, lanes 13-18) as indicated. Then cells were stimulated or not for another 5 min with 100 nmol/l insulin. PKB Ser(473) phosphorylation changes were monitored and normalized as described in the legend to Figure 2. *, PKB phosphorylation significantly ($p \leq 0.05$) different from that found in cells stimulated with insulin in absence of TLCS and inhibitors. §, PKB phosphorylation significantly ($p \leq 0.05$) different from that in insulin-stimulated cells pre-treated with TLCS only. (n=6-8). (B) Efficacy of pharmacological MAP-kinase pathway inhibition. Hepatocytes were exposed to TLCS for 6 h in absence or presence of PD98059 (PD, 20 $\mu\text{mol/l}$) or SP600125 (50 $\mu\text{mol/l}$) as indicated (lanes 1-3 and 4-6). Where indicated cells were stimulated with 100 nmol/l insulin for another 5 min. Alternatively, hepatocytes were stimulated with anisomycin (25 $\mu\text{mol/l}$, 30 min) in presence or absence of SB203580 (20 $\mu\text{mol/l}$, lanes 7-9). Cell lysates were analyzed for the presence of Thr/Tyr-phosphorylated Erk-1/Erk-2 and JNK1, phosphorylated MAPKAP2 and overall Erk-1/Erk2, JNK1 and MAPKAP2. Representatives of at least three independent experiments are shown. *, Phosphorylation of the respective kinase significantly ($p \leq 0.05$) different from that found in absence of the inhibitor under the indicated condition.

rylation by TLCS, but consistently decreased overall protein tyrosine nitration below basal levels (Figure 7, lines 55-60), indicating the efficacy of the compound. Finally, AG1478, a specific EGF receptor tyrosine kinase inhibitor that blocks TLCS-induced activation of CD95 and apoptosis [7, 32], did not restore PKB phosphorylation by insulin in presence of TLCS. The data suggest that a signaling branch different from that mediating apoptosis accounts for the TLCS-induced downregulation of insulin signaling via PKB.

TLCS impairs the tyrosine phosphorylation of IR β by insulin

As shown in Figure 8, a 6 h pre-treatment of cultured hepatocytes with TLCS impaired Tyr(1158) phosphorylation of the insulin receptor β subunit (IR β) by insulin, whereas overall IR β expression remained stable in presence of TLCS. Whereas Gö6850 potentially antagonized the suppression of insulin-induced IR β phosphorylation by TLCS (lines 1-6), rapamycin was ineffective (lines 7-12). Further, neither dibutyryl-cAMP and EGCG, nor AG1478 restored IR β tyrosine phosphorylation by insulin in presence of TLCS (lines 13-30). It is suggested that Gö6850-sensitive PKC isoforms are involved in mediating the suppression of the IR β response to insulin by TLCS.

Fig. 7. Pharmacological characterization of the suppression of insulin-induced PKB phosphorylation by TLCS in cultured rat hepatocytes: Hepatocytes were pretreated for 6 h with TLCS (100 $\mu\text{mol/l}$) in presence of vanadate (Van, 500 $\mu\text{mol/l}$), okadaic acid (OA, 5 nmol/l), FMK001 (40 $\mu\text{mol/l}$), dibutyryl-cAMP (100 $\mu\text{mol/l}$), Gö6850 (10 $\mu\text{mol/l}$), rapamycin (500 nmol/l), AY9944 (5 $\mu\text{mol/l}$), PKC ζ -inhibitory peptide (20 $\mu\text{mol/l}$, PKC ζ -I), apocynin (300 $\mu\text{mol/l}$), epigallocatechin gallate (EGCG, 30 $\mu\text{mol/l}$) AG1478 (5 $\mu\text{mol/l}$), as indicated. Then, cells were stimulated or not with 100 nmol/l insulin for another 5 min. PKB Ser(473) phosphorylation changes were monitored and normalized as described in the legend to Figure 2. *, PKB phosphorylation significantly ($p \leq 0.05$) different from that in cells stimulated with insulin in the absence of TLCS and inhibitors. §, PKB phosphorylation significantly ($p \leq 0.05$) different from that in insulin-stimulated cells following pretreatment with TLCS only (n=3-7).

TUDC antagonizes the suppression of insulin-induced PKB phosphorylation by TLCS

TUDC is a therapeutically useful hydrophilic bile acid that protects the liver from different kinds of stress [3, 16, 22]. In order to address the effect of TUDC on the suppression of insulin-induced PKB phosphorylation by TLCS, both bile acids were co-administered. As shown in Figure 9, TUDC prevented the suppression of insulin-induced PKB phosphorylation by TLCS (lines 1-6). TC was less efficient in this regard (lines 7-12), whereas taurine had no significant effect on PKB phosphorylation by insulin in presence of TLCS (lines 13-18). TUDC, TC and taurine combined with TLCS did not increase PKB phosphorylation in the absence of insulin.

The finding that rapamycin prevented the TLCS-induced insulin resistance at the level of PKB phosphorylation (Figure 7) may suggest that dibutyryl-cAMP, Gö6850 and TUDC abolished this TLCS effect via a downregulation of mTOR-dependent signaling. In order to address the effect of TLCS on mTOR signaling, the phosphorylation state of the p70 ribosomal S6 protein kinase (p70 S6-kinase) and the eukaryotic initiation factor 4E binding protein (4E-BP1), which are downstream targets of mTOR [33], was examined. Changes of p70 S6-kinase phosphorylation were visualized in Western blots by antibodies recognizing Thr421/Ser424-phosphorylated p70 S6-kinase and electrophoretic mobility shifts of p70S6-kinase, respectively. Changes of 4E-BP1 phosphoryla-

tion were visualized by Western blot detection of 4E-BP1 electrophoretic mobility shifts. As shown in Figure 10, TLCS was without effect on the phosphorylation state of p70 S6-kinase but slightly decreased 4E-BP1 phosphorylation (lines 1, 2, 5, 6). As expected, rapamycin led to a pronounced de-phosphorylation of p70 S6-kinase and 4E-BP1, as shown by a decrease of Thr421/Ser424-phosphorylated p70 S6-kinase immunoreactivity and by an increased electrophoretic mobility of Thr421/Ser424-phosphorylated and overall p70 S6-kinase and 4E-BP1, respectively (lines 3, 4 and 7). Effects similar to those induced by rapamycin were produced by dibutyryl-cAMP (line 11) and, to a lesser extent, by Gö6850 (line 8). Although the PKC ζ -inhibitory pseudosubstrate peptide decreased the levels of Thr421/Ser424-phosphorylated p70 S6-kinase, the electrophoretic mobilities of Thr421/Ser424-phosphorylated p70 S6-kinase and 4E-BP1 were similar to those found under the control condition (compare lines 1, 2, 5, 6 and 9). Of interest, TUDC, like EGCG, neither affected the level of Thr421/Ser424-phosphorylated p70 S6-kinase, nor increased the electrophoretic mobility of p70 S6-kinase and 4E-BP1 (compare lines 1, 2, 5, 6 with 10 and 11). The data suggest that an inhibition of basal mTOR signaling could contribute to the restoration of insulin-induced PKB phosphorylation in the presence of TLCS by rapamycin, Gö6850 and dibutyryl-cAMP but not by TUDC.

Fig. 8. Effect of TLCS on insulin-induced IR β tyrosine phosphorylation in cultured rat hepatocytes: Hepatocytes were pretreated for 6 h with TLCS (100 μ mol/l) in presence of rapamycin (Rapa, 500 nmol/l), Gö68650 (10 μ mol/l), dibutyryl-cAMP (dBcAMP, 100 μ mol/l), epigallocatechin gallate (EGCG, 30 μ mol/l) and AG1478 (5 μ mol/l), as indicated. Then, cells were stimulated with insulin (100 nmol/l) or not for another 5 min. IR β Tyr(1158) phosphorylation was monitored according to the procedure given in the legend to Figure 2. IR β phosphorylation was normalized to overall IR expression levels and IR β phosphorylation by insulin without pre-treatment with TLCS and inhibitors was set to 1. *, IR β phosphorylation significantly ($p \leq 0.05$) different from that found in cells stimulated with insulin in absence of TLCS and inhibitors. §, IR β phosphorylation significantly ($p \leq 0.05$) different from that in cells stimulated with insulin following pre-treatment with TLCS only (n=3-4).

Fig. 9. Effect of TUDC on the TLCS-induced suppression of insulin-induced PKB phosphorylation in cultured rat hepatocytes: Hepatocytes were pretreated for 6 h under serum-free conditions with TLCS (100 μ mol/l) or TLCS (100 μ mol/l) combined with TC (100 μ mol/l), TUDC (100 μ mol/l) or taurine respectively, as indicated. Taurine was already added 24 h before the switch to serum-free medium. Then cells were stimulated or not with insulin (100 nmol/l) for another 5 min. PKB Ser(473) phosphorylation changes were monitored as described in the legend to Figure 2. *, PKB phosphorylation significantly ($p \leq 0.05$) different from that in cells stimulated with insulin in absence of TLCS and TUDC. §, PKB phosphorylation significantly ($p \leq 0.05$) different from that in cells stimulated with insulin following pre-treatment with TLCS only (n=6-8).

TUDC antagonizes the inhibition of insulin-induced IR β phosphorylation by TLCS

The influence of TUDC on the impairment of insulin-induced IR β Tyr(1158) phosphorylation by TLCS was examined (Figure 11). TUDC significantly increased the IR β phosphorylation by insulin in TLCS-treated hepatocytes but neither in combination with TLCS, nor by itself increased IR phosphorylation in the absence of insulin (lines 1-10). As shown in Figure 11 (lines 11-14), the partial restoration of insulin-induced IR β phosphorylation by TUDC in TLCS-treated hepatocytes was insensitive to the MAP-kinase pathway inhibitors SB203580 and PD98059 (lines 11, 12), the PKA inhibitor H89 (line

14), and the PI 3-kinase inhibitors LY294002 (line 13) and wortmannin (100 nmol/l, not shown). The PI 3-kinase inhibitors effectively prevented PKB phosphorylation by insulin in presence of TLCS plus TUDC (data not shown). The findings suggest that neither PI 3-kinase, nor MAP-kinases and PKA mediate the TUDC-induced restoration of IR β phosphorylation by insulin in TLCS-treated hepatocytes.

Fig. 10. Effects of signal transduction inhibitors on mTOR-dependent signaling in TLCS-treated cultured rat hepatocytes: Hepatocytes were treated for 6 h with TLCS (100 $\mu\text{mol/l}$) alone or combined with rapamycin (500 nmol/l), Gö6850 (10 $\mu\text{mol/l}$), PKC ζ -inhibitory peptide (PKC ζ -I, 20 $\mu\text{mol/l}$), TUDC (100 $\mu\text{mol/l}$), dibutyryl-cAMP (dBcAMP, 100 $\mu\text{mol/l}$) or epigallocatechin gallate (EGCG, 30 $\mu\text{mol/l}$), as indicated. Cell lysates were analyzed for the presence of Thr(421)/Ser(424)-phosphorylated p70 S6 kinase, overall p70 S6 kinase and eukaryotic initiation factor 4E binding protein (4E-BP1). For monitoring of 4E-BP1 electrophoretic mobility shifts lysates from leucine-infused rat liver [17] were used as a positive control (lane 13). The anti-p70 S6 kinase antibodies display some cross reactivity to the p85 S6 kinase. For statistical analysis changes of Thr(421)/Ser(424)-phosphorylated P70S6-kinase, P70S6-kinase, 4E-BP1 γ and 4E-BP1 α expression were monitored by densitometry. Thr(421)/Ser(424)-P70 S6 kinase expression was normalized to overall P70S6 kinase expression and P70S6 kinase phosphorylation in absence of TLCS (lines 1, 5) was set to 1. For estimation of 4E-BP1 phosphorylation changes the ratio of 4E-BP1 γ to 4E-BP1 α was calculated. *, P70S6 kinase Thr(421)/Ser(424) or 4E-BP1 phosphorylation, respectively, was significantly ($p \leq 0.05$) decreased compared to that found under the control condition (TLCS absent, lines 1, 5). §, 4E-BP1 phosphorylation differs significantly ($p \leq 0.05$) from that found in presence of TLCS in the absence of inhibitors (lines 2, 6). The figure summarizes data from 3 independent experiments.

TLCS impairs proteolysis inhibition by insulin in perfused rat liver

Insulin in the perfused rat liver potently increased PKB Ser(473) phosphorylation [17] and in a PI 3-kinase-dependent manner stimulated K^+ uptake, hepatocyte swelling and inhibition of autophagic proteolysis [34]. Here, the effect of TLCS (10 $\mu\text{mol/l}$) on insulin-induced K^+ uptake, hepatocyte swelling and proteolysis inhibition was studied in perfused rat liver. In the absence of TLCS, insulin (35 nmol/l) induced a net K^+ uptake, hepatocyte swelling and proteolysis inhibition (Figure 12) by $+5.7 \pm 0.3 \mu\text{mol/g}$ ($n=21$), $12.5 \pm 1.4 \%$ ($n=14$) and $21.5 \pm 1.7 \%$ ($n=4$), respectively. As shown in Figure 12, TLCS by itself inhibited proteolysis by $17.0 \pm 2.0 \%$ ($n=5$). However, in presence of TLCS the insulin-induced net K^+ uptake, hepatocyte swelling and proteolysis inhibition were significantly decreased to $+2.1 \pm 0.3 \mu\text{mol/g}$ ($n=4$), $+2.5 \pm 1.2 \%$ ($n=4$) and $4.2 \pm 1.9 \%$ ($n=5$), respectively. The residual proteolysis inhibition in the presence of TLCS is reversible upon removal of insulin (Figure 12), indicating a well-preserved viability of the liver. The data suggest that TLCS impairs PI 3-kinase-dependent insulin signaling in perfused rat liver.

Hepatic PKB phosphorylation in bile duct-ligated rats

PKB phosphorylation was examined in the livers from bile duct-ligated rats, which were used as an *in vivo* model of cholestasis. As shown in Figure 13, bile duct ligation induced a prograding PKB dephosphorylation, which was about 50 % in livers from 7 days bile duct-ligated rats ($p=0.007$). The data indicate a suppression of hepatic PI 3-kinase/PKB signaling under cholestatic conditions *in vivo*.

Fig. 11. Effect of TUDC on the TLCS-induced suppression of insulin-induced IR β phosphorylation in cultured rat hepatocytes: Hepatocytes were pretreated for 6 h with TLCS (100 μ mol) or TLCS (100 μ mol/l) combined with TUDC (100 μ mol/l) as indicated without further additions (lines 1-9) or in presence of SB203580 (20 μ mol/l), PD98059 (20 μ mol/l), LY294002 (50 μ mol/l) and H89 (10 μ mol/l), as indicated. Then cells were stimulated or not with insulin (100 nmol/l) for another 5 min. Cell lysates were assayed for Tyr(1158)-phosphorylated IR β and overall IR β , respectively. IR β phosphorylation was normalized to overall IR β expression and IR β phosphorylation by insulin without pre-treatment with TLCS and inhibitors was set to 1. *, IR β phosphorylation significantly ($p \leq 0.05$) different from that in cells stimulated with insulin in absence of TLCS and inhibitors. §, IR β phosphorylation significantly ($p \leq 0.05$) different from that in cells stimulated with insulin following pre-treatment with TLCS only ($n=3$).

Discussion

This study shows that TLCS and GCDC but not TC and TUDC impair insulin-induced PKB Ser(473) phosphorylation in cultured rat hepatocytes (Figure 1). Moreover, insulin-induced Tyr(1158) phosphorylation and PI 3-kinase activation by insulin were blunted by TLCS (Figures 5 and 8). In line with this, the insulin-induced K⁺-uptake, hepatocyte swelling and proteolysis inhibition was inhibited by TLCS (Figure 12) and in livers from bile duct-ligated rats basal PKB Ser(473) phosphorylation was decreased (Figure 13). TUDC potentially counteracted

Fig. 12. Effect of TLCS on insulin-induced autophagic proteolysis inhibition in perfused rat liver: Proteolysis was monitored as described in the Materials and Methods. Insulin (35 nmol/l) was infused under control conditions ($n=4$) (A) or following a 60 min pre-perfusion with 10 μ mol/l TLCS ($n=5$) (B).

the suppression of insulin-induced phosphorylation of IR β and PKB in TLCS-treated hepatocytes.

Multiple mechanisms may operate in the suppression of insulin signaling by TLCS. Hydrophobic bile acids activate a panel of G δ 6850-sensitive PKC isoforms including PKC α , β , δ and ϵ [35-37]. As shown in this paper, G δ 6850 effectively increased the insulin-induced tyrosine phosphorylation of IR β and Ser(473) phosphorylation of PKB in TLCS-treated hepatocytes (Figures 7, 8), indicating that PKC is involved in the suppression of insulin-induced phosphorylation of IR β and PKB by TLCS. In fact, a contribution of G δ 6850-sensitive PKC

Fig. 13. PKB phosphorylation in livers of bile duct-ligated rats: Rats were bile duct-ligated for 1, 3 and 7 days, respectively. Samples from livers of bile duct-ligated rats and the corresponding sham-operated rats were analyzed by Western blot for the presence of Ser(473)-phosphorylated PKB, overall PKB and glyceraldehyde 3-phosphate dehydrogenase (GAPDH), respectively. Changes in Ser(473)-phosphorylated PKB were monitored by densitometry and normalized to overall PKB expression. *, PKB phosphorylation in livers of the bile duct-ligated animals significantly ($p \leq 0.05$) different to that found in the corresponding sham-operated animals. §, hepatic PKB phosphorylation significantly ($p \leq 0.05$) different from that found in livers 1 day after bile duct ligation. Each group consisted of 4 animals.

isoforms including α , β , δ and ϵ to IR β inhibition has been well established in different settings of cellular insulin resistance (e.g. [38, 39]).

Interestingly, rapamycin and dibutyryl-cAMP increased the insulin-induced PKB Ser(473) phosphorylation, but not IR β tyrosine phosphorylation in TLCS-treated hepatocytes (Figures 7, 8). Similar to rapamycin and consistent with earlier reports [40, 41] dibutyryl-cAMP decreased the basal phosphorylation of both, p70 S6-kinase and 4E-BP1 (Figure 10), indicating a downregulation of mTOR signaling by both compounds. In different insulin target tissues including the liver, a sustained activation of mTOR and p70 S6-kinase was shown to trigger insulin

Fig. 14. TLCS-induced insulin resistance in rat hepatocytes: a working hypothesis: The in part hypothetical scheme depicts that TLCS (6h pre-treatment) interferes with insulin-induced IR β tyrosine phosphorylation and activation of IRS1-associated PI 3-kinase and PKB. The PKC inhibitor Gö6850 and the hydrophilic bile acid TUDC restore the insulin-induced IR β tyrosine phosphorylation and PKB Ser(473) phosphorylation in TLCS-treated hepatocytes, whereas rapamycin and dBcAMP restore the insulin-induced PKB Ser(473) phosphorylation only. Gö6850 and dBcAMP, like rapamycin, decrease mTOR-dependent signals, which may release IRS from tonic inhibition and thereby compensate for inhibitory effects of TLCS, which are independent from mTOR. (Pro)apoptotic TLCS signals (see Figure 13 in Ref. 32) are apparently not involved in the inhibition of insulin-induced IR β tyrosine- and PKB Ser(473) phosphorylation by insulin in the cultured hepatocytes.

resistance by serine phosphorylation of IRS proteins, leading to an impaired recruitment of PI 3-kinase to IRS and consequently PKB activation [42]. A decrease of IRS1-associated PI 3-kinase activity was indeed observed following insulin stimulation of TLCS-treated hepatocytes (this paper, Figure 5). However, TLCS by itself did not increase phosphorylation of p70 S6-kinase and 4E-BP1 (Figure 5), indicating that mTOR may play a permissive role in the suppression of PKB phosphorylation by TLCS. Inhibition of mTOR signaling by rapamycin and dibutyryl-cAMP might relieve IRS from tonic inhibition and thereby allow a compensation for mTOR-independent suppression of insulin signaling by TLCS.

How TUDC increases insulin-induced IR β and PKB phosphorylation in TLCS-treated hepatocytes is currently unclear. TUDC was recently shown to improve insulin sensitivity in diabetic animals, which was attributed to an inhibition of endoplasmic reticulum (ER) stress-related JNK activation [43]. Hydrophobic bile salts in hepatocytes increase ER stress [44] and potentially activate JNK (e.g. [16], this paper, Figure 6B) and TUDC inhibited

TLCS-induced JNK activation [16]. However, inhibition of TLCS-induced JNK activation by SP600125 did not improve the PKB sensitivity to insulin (this paper, Figure 6), indicating that JNK activation is not involved in the suppression of insulin-induced PKB Ser(473) phosphorylation by TLCS and that JNK inhibition plays no major part in the improvement in insulin sensitivity by TUDC in TLCS-treated hepatocytes. TUDC did not affect the phosphorylation state of p70 S6-kinase or 4E-BP1 (Figure 10), suggesting that modulation of mTOR signaling is also not involved. On the other hand, TUDC was reported to antagonize the activation of Gö6850-sensitive PKC isoforms by hydrophobic bile acids [37,45]. Therefore, it seems well conceivable that TUDC restores insulin-induced IR β and PKB phosphorylation via inhibition of TLCS-induced PKC activation. However, the possibility that competition of TUDC with TLCS for uptake accounts for the TUDC-induced recovery of insulin-induced IR β and PKB phosphorylation can not be excluded, although such a competition was considered unlikely to play a role in the TLCS-induced protection of rat hepatocytes from TLCS- or GCDC-induced apoptosis [16, 22].

Autophagic proteolysis inhibition by insulin in the perfused rat liver is mediated by a PI 3-kinase-dependent net K⁺-uptake and hepatocyte swelling [34, 46]. The latter is coupled to proteolysis inhibition by an integrin-dependent signaling mechanism [17, 47]. The TLCS-

induced impairment of insulin-induced K⁺-uptake, hepatocyte swelling and proteolysis inhibition (Figure 12) may be due to inhibition of PI 3-kinase signaling by TLCS. Further, in perfused rat liver TLCS stimulated hepatocyte shrinkage [48]. Hepatocyte shrinkage as induced by hyperosmolarity prevented insulin-induced K⁺ uptake and hepatocyte swelling [34] and may also contribute to the TLCS-induced insulin resistance in perfused rat liver.

Insulin resistance is a widespread condition in cholestatic liver disease and the content of hydrophobic bile acids in the circulating bile acid pool is increased [49]. From the study presented here it is concluded that hydrophobic bile acids in hepatocytes produce insulin resistance by a PKC-dependent suppression of insulin-induced IR β phosphorylation and the PI 3-kinase/PKB path, which can in part be compensated by a decrease of basal mTOR activity (Figure 14). Further studies might elucidate the impact of bile acid retention on insulin resistance in liver disease.

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