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Prevention of *Clostridium sordellii* Lethal Toxin-Induced Apoptotic Cell Death by Tauroursodeoxycholic Acid[†]

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ABSTRACT: Virulent strains of *Clostridium sordellii* cause gangrenous myonecrosis in humans. The released lethal toxin (TcsL) and hemorrhagic toxin (TcsH) are regarded as the major virulence factors. TcsL inactivates low molecular weight GTP-binding proteins of the Rho/Ras subfamilies by monoglucosylation. In cultured cell lines, glucosylation, i.e., inactivation of Rho/Ras proteins, results in actin reorganization ("cytopathic effect") and apoptotic cell death ("cytotoxic effect"). Apoptotic cell death induced by TcsL is suggested to be based on inhibition of the phosphoinositide 3-kinase (PI3K)/Akt-survival pathway. In this study, we analyze the critical role of PI3K/Akt signaling in TcsL-induced apoptosis using the antiapoptotic bile acid tauroursodeoxycholic acid (TUDCA) as the pharmacological tool. TUDCA preserved the TcsL-induced decrease of the cellular level of phospho-Akt, suggesting that TUDCA activated PI3K/Akt signaling downstream of inhibited Ras signaling. TcsL-induced apoptosis was prevented by TUDCA treatment. The antiapoptotic effect of TUDCA was abolished by the PI3K inhibitor LY294002 and the Akt inhibitor, showing that the antiapoptotic effect depends on PI3K/Akt signaling. Inhibition of Ras/Rho signaling by TcsL resulted in activation of p38 MAP kinase. Inhibition of p38 MAP kinase by SB203580 protected cells from TcsL-induced apoptosis. TUDCA induced activation of p38 MAP kinase as well, an aspect of the TUDCA effects that most likely did not contribute to its antiapoptotic activity. Due to its antiapoptotic activity, TUDCA is under investigation for its potential application as a therapeutic modulator of apoptosis-related diseases. TUDCA may represent a new concept for the treatment of disease associated with toxigenic *C. sordellii*.

Clostridium sordellii is an anaerobic, spore-forming microbe. Virulent *C. sordellii* strains cause enteritis and enterotoxemia in sheep and cattle as well as myonecrosis and gangrene in humans. Pathogenic strains of *C. sordellii* produce up to seven virulence factors, including lethal toxin (TcsL),[1] hemorrhagic toxin (TcsH), a hemolysin, a DNase, a collagenase, and a lysolecithinase (*1*, *2*). TcsL and TcsH are regarded as the major virulence factors in *C. sordellii*-associated diseases. Intradermal injection of these protein toxins into animals results in local necrosis, progressive edema due to local and systemic vascular permeability, and death, a process similar to that seen in some *C. sordellii* infections in humans (*3*). Many toxigenic *C. sordellii* strains, however, solely produce TcsL but not TcsH (*4*).

TcsL and TcsH are related to toxin A (TcdA) and toxin B (TcdB) from *Clostridium difficile*, the causative agents of the *C. difficile*-associated diarrhea (CDAD) and its severe form the pseudomembranous colitis (PMC) (*5*, *6*). Due to their inherent glucosyltransferase activity, these toxins have been classified as "clostridial glucosylating toxins" (*5*). These toxins are single-chained protein toxins with an AB toxin-like structure. The C-terminal delivery domain harbors domains required for receptor binding, membrane translocation, and autocatalytic processing, allowing target cell entry by receptor-mediated endocytosis (*7*, *8*). The N-terminal glucosyltransferase domain causes glucosylation and thereby inactivation of low molecular weight GTP-binding proteins of the Rho/Ras subfamilies. While TcdA, TcdB, and TcsH specifically glucosylate Rho, Rac, and Cdc42, TcsL inactivates Rac and Cdc42 as well as the Ras family proteins (H/K/N/R)Ras, Rap(1,2), and Ral (*4*, *6*, *9*, *10*). On cultured cell lines, the glucosylating toxins cause actin reorganization ("cytopathic effect") and apoptotic cell death ("cytotoxic effect"). Glucosylation of Rac1 has recently been suggested to be critical for actin reorganization induced by the glucosylating toxins (*11*, *12*).

Many cell surface receptors induce the production of second messengers that activate phosphoinositide 3-kinase (PI3K). PI3K generates phosphorylated phosphatidylinositides (PI-3,4-P2 and PI-3,4,5-P3) in the cell membrane that bind to the amino-terminal pleckstrin homology domain of the serine/threonine kinase Akt. Activated Akt promotes cell survival through suppression of apoptosis by (i) phosphorylation of the Bad component of the Bad/Bcl-$_{XL}$ complex and (ii) degradation of IKK-α that ultimately leads to NF-kB activation and cell survival (*13*).

The endogenous hydrophilic bile acid ursodeoxycholic acid and its taurine-conjugate tauroursodeoxycholic acid (TUDCA) act antiapoptotic, likely through activation of PI3K/Akt signaling downstream of apoptosis-inducing stimuli (*14*, *15*). TUDCA is an approved drug for the treatment of cholestasis and biliary cirrhosis (*16*). Inhibition of PI3K/Akt signaling has been recently suggested to be critical for apoptosis induced by the Ras-glucosylating TcsL (*10*, *17*). In this study, we show that TUDCA

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[1]Abbreviations: PDK1, phosphoinositide-dependent protein kinase 1; PI3K, phosphoinositide 3-kinase; RBL cells, rat basophilic leukemia cells; TcdA, toxin A from *Clostridium difficile* strain VPI10463; TcdB, toxin B from *C. difficile* strain VPI10463; TcsH, hemorrhagic toxin from *Clostridium sordellii*; TcsL, lethal toxin from *C. sordellii*; TUDCA, tauroursodeoxycholic acid.

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preserved PI3K/Akt activity in TcsL-treated cells and thus prevented TcsL-induced apoptosis but not apoptosis induced by the related TcdA/TcdB. This study suggests that TUDCA may represent a promising candidate for the treatment of disease associated with toxigenic *C. sordellii*.

MATERIALS AND METHODS

Materials. Sodium tauroursodeoxycholate (TUDCA) was obtained from Sigma. LY294002, SB203580, and the Akt inhibitor (1L6-hydroxymethyl-*chiro*-inositol 2-(*R*)-2-*O*-methyl-3-*O*-octadecyl-*sn*-glycerocarbonate) were from Calbiochem. Anti-β-actin (clone AC-40) was from Sigma. Anti-RhoB (C-5) sc8048 was purchased from Santa Cruz Biotechnology. Anti-Ras (27H5) and anti-phospho-Akt (Ser 473) (193H12) were from Cell Signaling. Anti-Rac1 (clone 102) was obtained from BD Transduction Laboratories. Horseradish peroxidase-conjugated secondary antibodies to mouse and rabbit immunoglobulin were from Rockland. Annexin V Alexa Fluor 488 conjugate was obtained from Invitrogen. The caspase-3 colorimetric assay was from R&D Systems. WST-1 (4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2*H*-5-tetrazolio]-1,3-benzenedisulfonate) was purchased from Roche. UDP-[^{14}C]glucose was purchased from Biotrend. Rac1 was recombinantly expressed as a GST fusion protein from *Escherichia coli* as described (*18*).

Toxin Purification. TcdA/TcdB and TcsL were purified from the *C. difficile* strain VPI10463 and the *C. sordellii* strain 6018 (identical to strain IP-82), respectively (*19*). In brief, a dialysis bag containing 900 mL of 0.9% NaCl in a total volume of 4 L of brain heart infusion (Difco) was inoculated with 100 mL of an overnight culture of *C. difficile* or *C. sordellii* and grown under microaerophilic conditions at 37 °C for 72 h. Proteins were precipitated from the culture supernatant by ammonium sulfate at 70% saturation. The precipitates were dialyzed against Tris-HCl buffer (pH 7.5) overnight and loaded onto a MonoQ column (Amersham Biosciences). The toxins were eluted with a NaCl gradient ranging from 0 to 800 mM and subsequently dialyzed against buffer (50 mM Tris-HCl, pH 7.5, 15 mM NaCl).

Cell Culture. J774A.1 macrophage-like cells were cultivated in RPMI 1640 medium (Biochrom) supplemented with 10% FCS, 100 μg/mL penicillin, 100 units/mL streptomycin, and 1 mM sodium pyruvate. Hela cells were cultivated in Dulbecco's modified Eagle medium (Gibco) supplemented with 10% FCS, 100 μg/mL penicillin, 100 units/mL streptomycin, and 1 mM sodium pyruvate. Both cell lines were maintained in 5% CO_2 at 37 °C and were passaged upon confluence.

Detection of Apoptotic Cells by Annexin V Staining. Cells were cultivated in a microtiter plate (24 well, tissue culture grade, flat bottom). Drugs and toxins were applied as indicated. Annexin V Alexa Fluor 488 was added into the medium. After 30 min incubation at 37 °C phosphatidylserine exposure was analyzed by fluorescence microscopy using a Zeiss Axiovert 200 M (Alexa Fluor 448: excitation, 470 nm; emission, 515 nm).

Caspase-3 Colorimetric Assay. Caspase-3 activity was analyzed using the Caspase-3 colorimetric assay (R&D Systems), according to the manufacturer's instructions. Cells were exposed to the toxins and drugs as indicated. Cells were lysed and subsequently incubated with the colorimetric substrate DEVD-pNA at 37 °C for 3 h. The caspase-3-dependent release of the reporter molecule pNA was quantified by photometry using a scanning multiwell spectrophotometer at 405 nm.

Detection of Viability by the WST-1 Test. The activity of cellular dehydrogenases was assessed using WST-1 (4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2*H*-5-tetrazolio]-1,3-benzenedisulfonate (Roche)). Cells were seeded in a microtiter plate (96 well, tissue culture grade, flat bottom). Drugs and toxins were applied as indicated. WST-1 was added directly into the medium, according to the manufacturer's instructions. The reduction of WST-1 to a formazan salt in viable cells was quantified using a scanning multiwell spectrophotometer at 450 nm.

Glucosylation of Recombinant Rac1. Rac1 (1 μM) dissolved in glucosylation buffer (50 mM Tris-HCl, pH 7.2, 150 mM NaCl, 100 mM KCl, 1 mM $MnCl_2$, 5 mM $MgCl_2$, 100 μg/mL BSA) was incubated with TcsL (1 μg/mL) in the presence of 10 μM UDP-[^{14}C]glucose. The reaction was terminated by the addition of Laemmli sample buffer after incubation at 37 °C for 20 min. Incorporated [^{14}C]glucose was detected by autoradiography.

Western Blot Analysis. Cells were cultivated in 35 mm dishes and treated with drugs and toxins as indicated. Sodium orthovanadate (1 mM) was added directly into the medium 30 min before harvesting the cells by scraping into Laemmli sample buffer. The resulting suspension was shaken for 10 min at 37 °C with 1400 rpm and subsequently sonified on ice. After incubation for 10 min at 95 °C, the obtained lysate was analyzed by SDS−PAGE. The separated proteins were subsequently transferred onto a nitrocellulose membrane using a tank blot gadget. Nonspecific binding sites were blocked with 5% (w/v) nonfat dried milk in Tris-buffered saline supplemented with 0.1% Tween (TBST) for 60 min. The membranes were washed three times with TBST for 10 min and thereafter incubated with primary antibody overnight at 4 °C. The membranes were washed again with TBST before applying the peroxidase-linked secondary antibody at room temperature for 60 min. The chemiluminescence reaction was performed using ECL Femto (Pierce).

Statistical Analysis. All data were evaluated using paired *t* tests with GraphPad software. *P*-values < 0.05 (*) and < 0.005 (**) were considered as statistically significant.

RESULTS

Inhibition of TcsL-Induced Apoptosis by TUDCA. TcsL induced apoptosis in macrophage-like J774A.1 cells and Hela cells, as analyzed in terms of phosphatidylserine exposure (Figure 1A). TcsL-induced apoptosis was prevented in cells pretreated with TUDCA in either cell line (Figure 1A). TUDCA alone did not induce apoptosis (Figure 1A). TcsL induced apoptosis in a concentration-dependent manner (Figure 1B). The antiapoptotic effect of TUDCA persisted even at high TcsL concentrations (Figure 1B). TUDCA prevented TcsL-induced phosphatidylserine exposure with comparable efficiency, regardless if J774A.1 cells were incubated with TUDCA 1 h prior, concomitantly, or 1 h after TcsL addition (Figure 1C). TUDCA treatment 2 h after TcsL treatment still caused a partial protective effect, while TUDCA treatment 4 h after TcsL treatment or later did not prevent phosphatidylserine exposure anymore (Figure 1C). These observations likely excluded that TUDCA acts through interference with TcsL uptake. To directly exclude that TUDCA interferes with glucosylation of the substrate proteins, a possible effect of TUDCA on Rac1 glucosylation was analyzed in J774A.1 cells and a cell-free system. Rac1 was exemplarily chosen as a sample substrate, as Rac1 represents a common substrate of TcsL and TcdA/TcdB (*12*, *18*). The kinetics of TcsL-induced

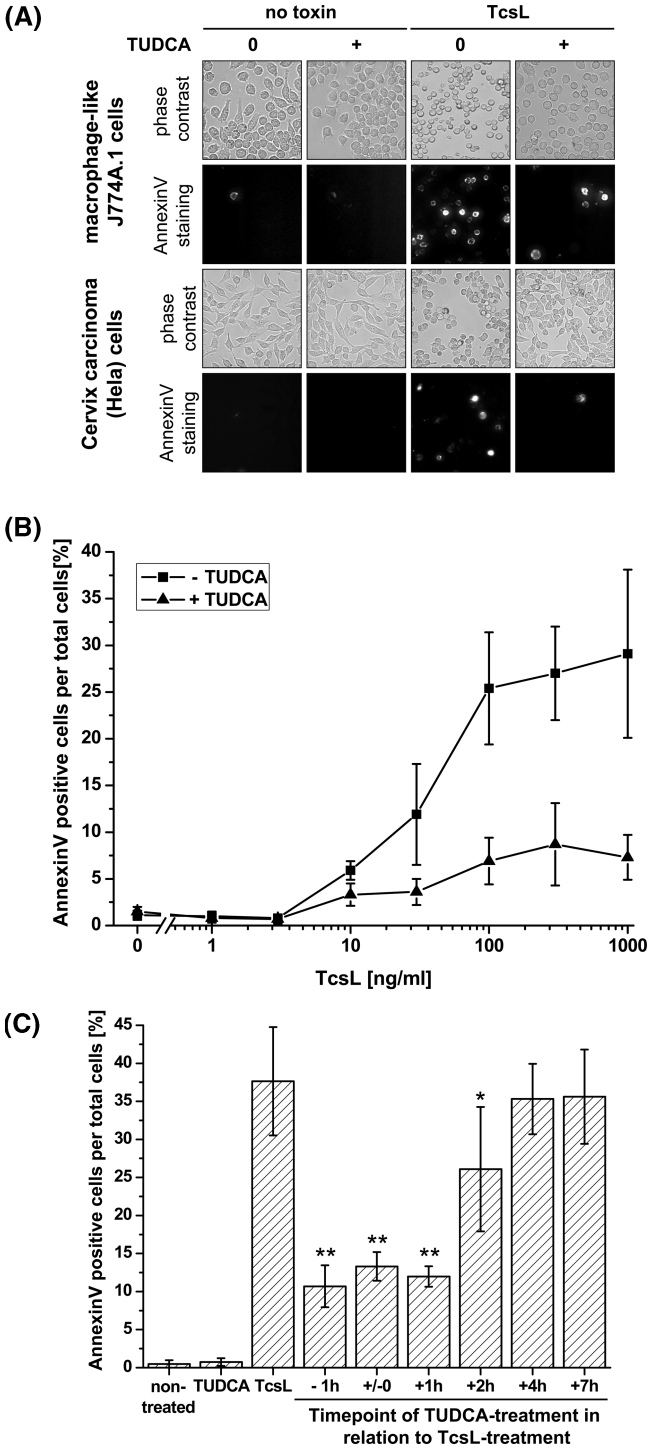

FIGURE 1: TUDCA prevents TcsL-induced apoptosis. (A) Phosphatidylserine exposure induced by TcsL was analyzed in J774A.1 cells and Hela cells either pretreated with TUDCA (300 μM) for 1 h or left untreated after TcsL treatment for 12 h. Annexin V-stained cells (lower panel) and phase contrast pictures (upper panel) are shown. (B) Phosphatidylserine exposure induced by increasing TcsL concentrations after 12 h of toxin treatment was quantified as the ratio of annexin V-positive per total cells. Results displayed represent the mean of three independent experiments. (C) Cells were treated with TUDCA (300 μM) either 1 h before treatment with TcsL (100 ng/mL) or added concomitantly with TcsL or applied at distinct time points as indicated post toxin treatment. Annexin V-positive cells were quantified after TcsL treatment for 16 h. Results displayed represent the mean of three independent experiments. *P*-values < 0.05 (*) and < 0.005 (**) were considered as statistically significant as compared with TcsL-treated cells.

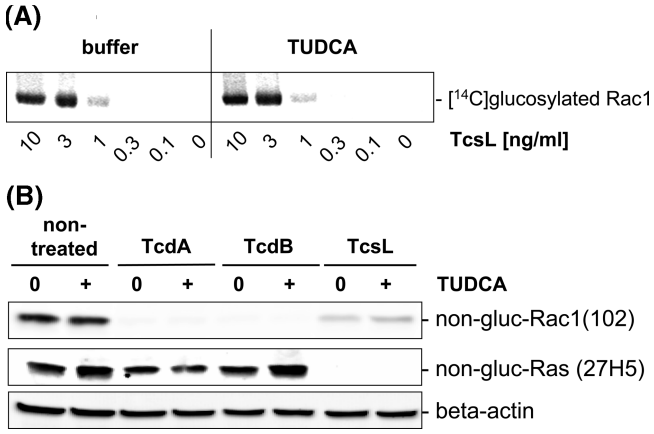

FIGURE 2: Effect of TUDCA on Rac1 glucosylation by the glucosylating toxins. (A) Rac1 was incubated in the presence of UDP-[^{14}C]-glucose and increasing concentrations of TcsL. Rac1 [^{14}C]glucosylation was recorded using autoradiography of the SDS−PAGE. (B) Cells either preincubated with TUDCA (300 μM) for 1 h or left untreated were exposed to either TcdA, TcdB, or TcsL (100 ng/mL each) for 12 h. The levels of nonglucosylated Rac1 and (H/K/N)Ras were analyzed by Western blotting using the glucosylation-sensitive Rac1-mAb(102) and Ras-mAb(27H5) antibodies.

[^{14}C]glucosylation of recombinant Rac1 were comparable in the presence or the absence of TUDCA (Figure 2A), excluding that TUDCA interfered with the glucosyltransferase activity of TcsL. Rac1 glucosylation was further analyzed in TcsL-treated J774A.1 cells using the glucosylation-sensitive antibody Rac1-mAb-(102) (*18*). The level of Rac1 apparently decreased in TcsL-treated cells compared to nontreated cells (Figure 2B), indicating Rac1 glucosylation. Rac1 was almost completely glucosylated regardless of TUDCA pretreatment (Figure 2B). Ras glucosylation in TcsL-treated cells was analyzed using the glucosylation-sensitive antibody Ras-mAb(27H5) that recognizes H-Ras, K-Ras, and N-Ras (Huelsenbeck and Genth, unpublished observation). (H/K/N)Ras were completely glucosylated in TcsL-treated cells regardless of TUDCA pretreatment. These findings showed that TUDCA treatment interfered with neither (H/K/N)Ras nor Rac1 glucosylation in TcsL-treated cells. The level of β-actin was affected by neither TcsL nor TUDCA treatment (Figure 2B). TUDCA thus interfered with Rac1 glucosylation neither in a cell-free system nor in TcsL-treated cells. These data suggest that TUDCA did not interfere with substrate glucosylation and cellular uptake of TcsL.

Activation of PI3K/Akt Signaling by TUDCA. TUDCA is classified as an activator of phosphatidylinositide 3′-OH kinase (PI3K)/Akt signaling. This notion was true in J774A.1 cells as well, as the level of phosphorylated, i.e., active, Akt increased upon TUDCA treatment (Figure 3A). The TUDCA-induced Akt activation as well as the residual Akt activity was inhibited by the PI3K inhibitor LY294002 (Figure 3A), confirming that Akt activity depended on PI3K.

The TcsL substrate proteins (H/K/N)Ras are upstream regulators of PI3K/Akt signaling. One must expect that treatment with the (H/K/N)Ras-glucosylating TcsL causes a decrease in the level of phosphorylated, i.e., active, Akt, which was in fact observed: The level of phosphorylated Akt decreased in TcsL-treated J774A.1 cells compared to nontreated cells (Figure 3B). Decreased levels of phosphorylated Akt have also been reported in TcsL-treated Hela cells (*17*) and rat basophilic leukemia (RBL) cells (*10*). The TcsL-induced decrease of Akt phosphorylation was prevented by pretreatment with TUDCA (Figure 3B). This

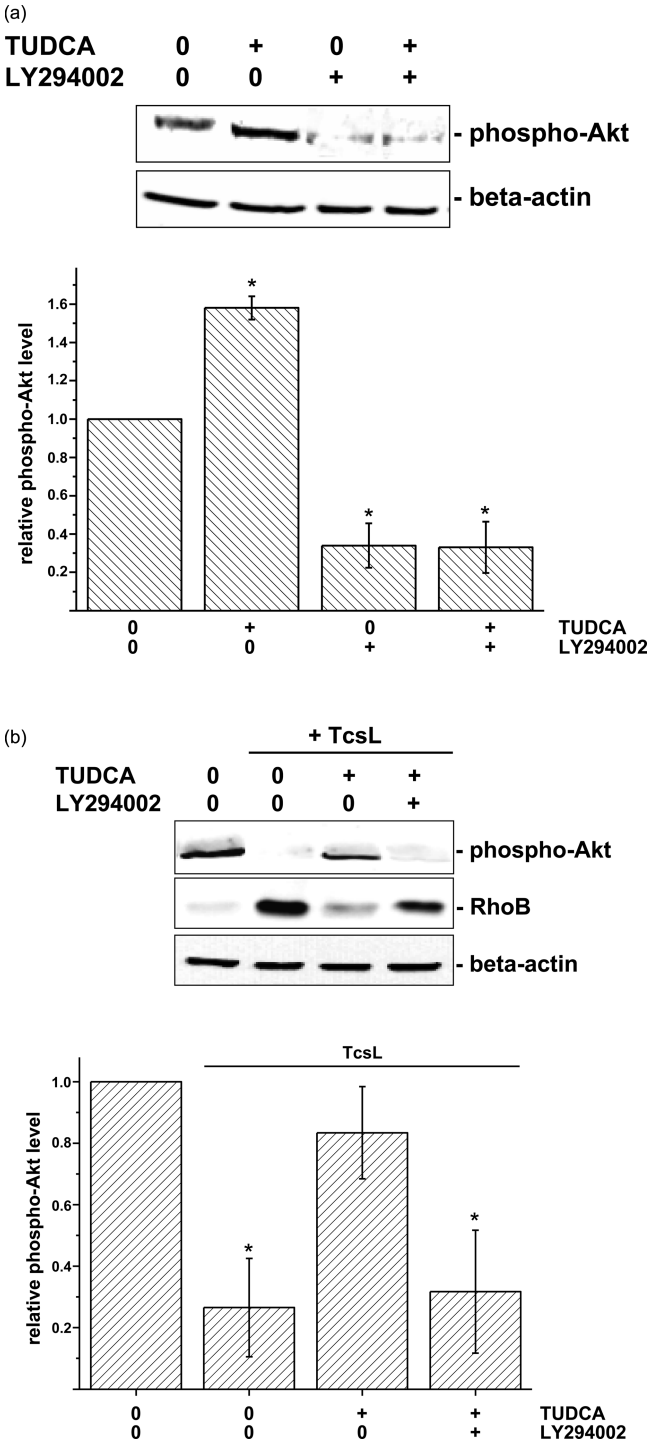

FIGURE 3: Effect of TUDCA on Akt phosphorylation. (A) Cells were treated with TUDCA (300 μM) and LY294002 (10 μM), and Akt phosphorylation (indicative of activation) was analyzed after 6 h by Western blotting. (B) Cells pretreated with TUDCA (300 μM) and LY294002 (10 μM) were exposed to TcsL (100 ng/mL) for 6 h. Signal intensities were densitometrically quantified and normalized to the β-actin signal. Akt phosphorylation in nontreated cells was set to 1.0. Data shown represent the mean of three independent experiments. P-values < 0.05 (*) were considered as statistically significant as compared with Akt phosphorylation from nontreated cells.

effect of TUDCA was abolished upon combined pretreatment of cells with TUDCA and LY294002 (Figure 3B). These findings suggest that TUDCA PI3K-dependently reactivated Akt in TcsL-treated cells.

The proapoptotic GTP-binding protein RhoB is one of the downstream targets of PI3K/Akt signaling. Especially, *rhoB* promoter activity is suppressed by (H/K/N)Ras in a PI3K/Akt-dependent manner (*20*). Increased RhoB expression upon TcsL treatment has been reported in murine fibroblasts (*21*) and RBL cells (*10*). To further provide evidence that TUDCA maintains PI3K/Akt signaling in TcsL-treated cells, expression of the downstream target RhoB was analyzed. TcsL induced RhoB expression in J774A.1 cells (Figure 3B). Pretreatment with TUDCA strongly reduced TcsL-induced RhoB expression, while RhoB expression was maintained in cells pretreated with both TUDCA and LY294002 (Figure 3B). These findings suggest that TUDCA PI3K-dependently restored suppression of proapoptotic RhoB in TcsL-treated cells.

Critical Role of PI3K/Akt Signaling in the Antiapoptotic Effect of TUDCA. We above showed that TUDCA prevented TcsL-induced apoptosis and preserved PI3K/Akt signaling in TcsL-treated cells (Figures 1 and 3). To check if the antiapoptotic effect of TUDCA depended on PI3K/Akt signaling, the antiapoptotic effect of TUDCA was analyzed for its responsiveness to inhibition by LY294002, an inhibitor of PI3K, or the Akt inhibitor. TcsL-induced apoptosis was analyzed in terms of phosphatidylserine exposure (Figure 4A), activation of the executioner caspase-3 (Figure 4B), and reduction of cell viability (Figure 4C). Treatment with TUDCA in fact prevented either aspect of apoptotic cell death (Figure 4). The antiapoptotic effect of TUDCA in TcsL-treated cells was inhibited by LY294002 or the Akt-inhibitor, showing that it depended on PI3K/Akt signaling. Neither the application of TUDCA, LY294002, or the Akt inhibitor alone (Figure 4A) nor simultaneous application of TUDCA and LY294002 or TUDCA and Akt inhibitor (data not shown) caused apoptosis. The responsiveness of the antiapoptotic effect of TUDCA to either LY294002 or the Akt inhibitor further excluded that TUDCA prevented TcsL uptake or activity.

Missing Sensitivity of TcdA-Induced Apoptosis to Prevention by TUDCA. To check whether the antiapoptotic effect of TUDCA is specific for TcsL, apoptosis induced by the related toxin A (TcdA) and toxin B (TcdB) from *C. difficile* was analyzed for responsiveness to TUDCA. Comparable to TcsL, TcdA caused phosphatidylserine exposure (Figure 5A,B) and a reduction of cell viability (Figure 5C) in J774A.1 cells. In contrast to TcsL, TUDCA prevented neither phosphatidylserine exposure nor a reduction of cell viability in TcdA-treated J774A.1 cells (Figure 5). Comparable to TcdA, TcdB-induced apoptosis of J774A.1 cells was not prevented by TUDCA (data not shown). The antiapoptotic effect of TUDCA was thus specific for TcsL. Comparable to above observation from TcsL-treated cells, TcdA/TcdB-induced Rac1 glucosylation was affected by TUDCA neither in a cell-free system (data not shown) nor in J774A.1 cells, as analyzed using the glucosylation-sensitive antibody Rac1-mAb(102) (Figure 2B). (H/K/N)Ras were expectedly not glucosylated by TcdA/TcdB, as no decrease in the detection of (H/K/N)Ras was observed when using the glucosylation-sensitive antibody Ras-mAb(27H5) (Figure 2B). The level of β-actin was not changed upon treatment with TcdA/TcdB and/or TUDCA (Figure 2B). These findings allow the conclusion that TUDCA did not prevent the glucosylation of the substrate proteins of TcdA/TcdB.

Role of p38 MAP Kinase in TcdA-/TcsL-Induced Apoptosis. TcdA-induced apoptosis is reported to be suppressed by inhibitors of p38 MAP kinase (*22*). In this line, TcdA-induced

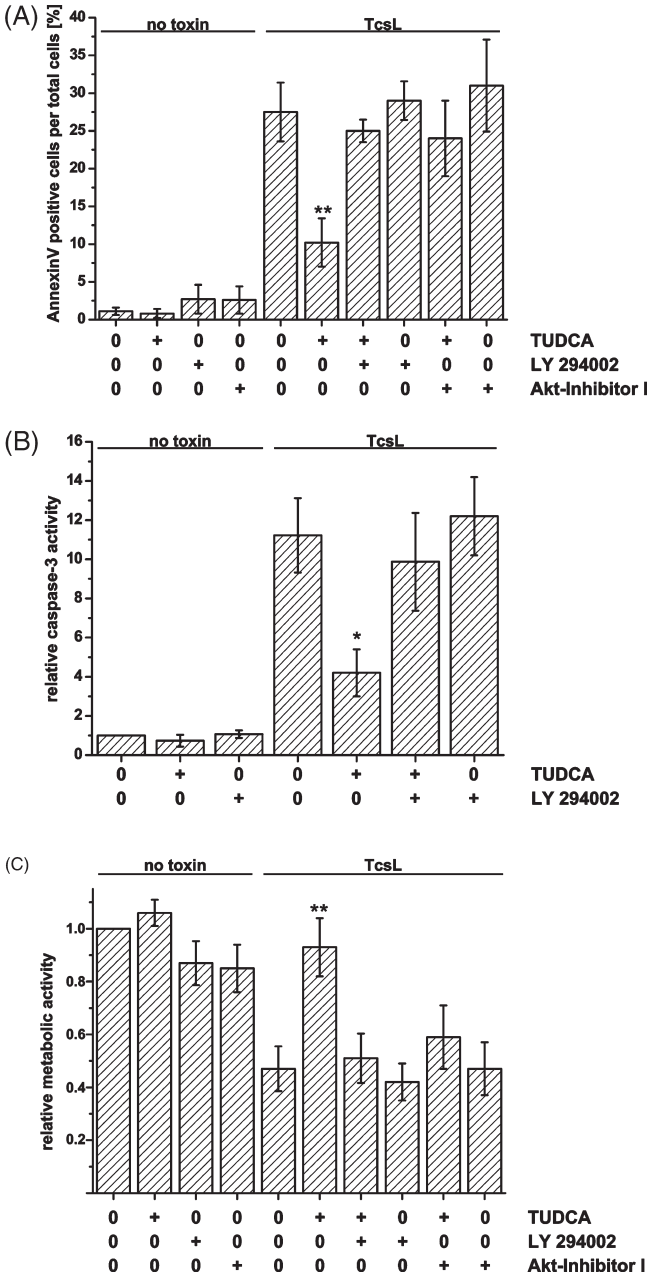

FIGURE 4: Effect of TUDCA on TcsL-induced apoptosis. J774A.1 macrophage-like cells pretreated with TUDCA (300 μM), LY294002 (10 μM), or the Akt inhibitor I (10 μM) as indicated were exposed to TcsL (100 ng/mL) for 12 h. (A) Phosphatidylserine exposure induced by increasing TcsL concentrations was quantified as the ratio of annexin V-positive per total cells. (B) Cells were lysed, and the activity of caspase-3 was analyzed using the caspase-3 colorimetric assay 6 h post toxin application. Caspase-3 activity in nontreated cells was set to 1.0. (C) Cell viability was analyzed using the cell proliferation reagent WST-1 24 h post toxin treatment. Cell viability of nontreated cells was set to 1.0. Results displayed represent the mean of three independent experiments. *P*-values < 0.05 (*) and < 0.005 (**) were considered as statistically significant as compared with cells treated with TcsL alone.

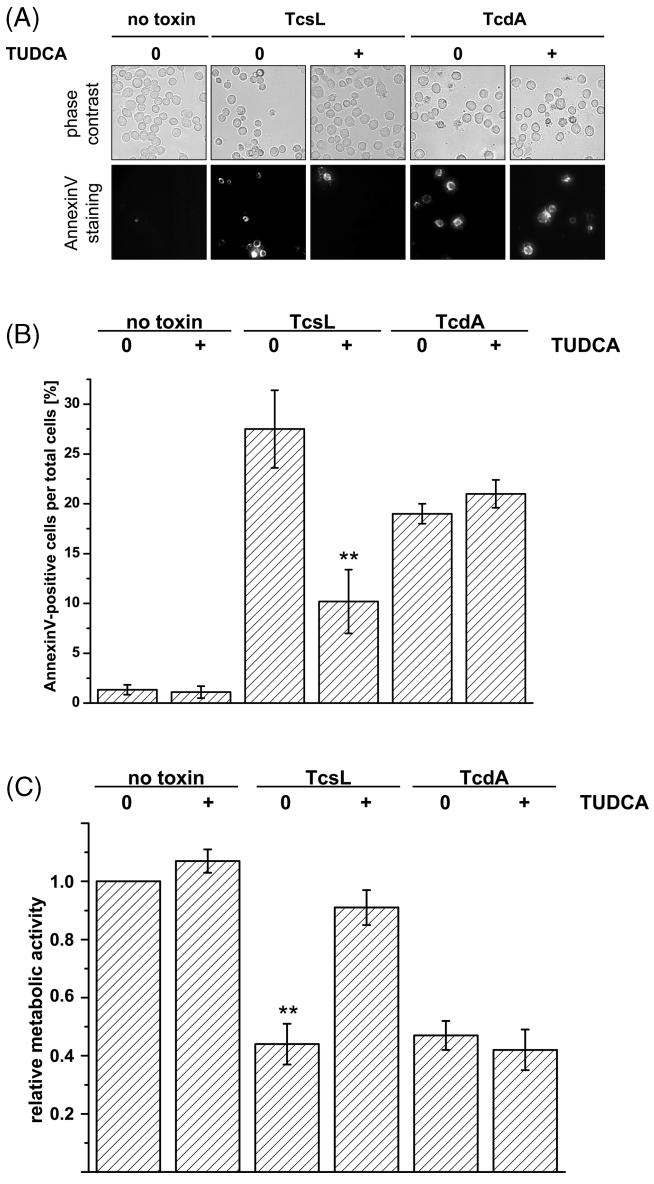

FIGURE 5: Effect of TUDCA on TcdA-induced apoptosis. (A) J774A.1 cells either pretreated with TUDCA (300 μM) or not were exposed to either TcdA or TcsL (100 ng/mL each) as indicated. Phosphatidylserine exposure was analyzed by fluorescence microscopy after 12 h of toxin treatment. (B) Toxin-induced apoptosis was quantified as the ratio of annexin V-positive per total cells. (C) TcdA-/TcsL-induced reduction of cell viability was analyzed in J774A.1 cells either pretreated with TUDCA (300 μM) for 1 h or not. The activity of cellular dehydrogenases was analyzed using the WST-1 test after toxin treatment for 24 h. Cell viability of nontreated cells was set to 1.0. The data represent the mean of three independent experiments. *P*-values < 0.005 (**) were considered as statistically significant as compared with cells treated with either TcsL or TcdA alone.

apoptosis in J774A.1 cells was susceptible to inhibition by SB203580, a pyridinylimidazole inhibitor of the p38 MAP kinase (Figure 6A to C). These observations excluded that the missing responsiveness of TcdA-induced apoptosis to TUDCA was based on a general insensitivity of TcdA-induced apoptosis to any treatment. TcsL-induced apoptosis also depended on p38 MAP kinase activity, as it was inhibited by SB203580 (Figure 6).

To investigate if the antiapoptotic activity of TUDCA involved inhibition of p38 MAP kinase, phosphorylation, i.e., activation of p38 MAP kinase, was analyzed in serum-starved J774A.1 cells. TUDCA induced activation of p38 MAP kinase (Figure 7A), corroborating published data (*23*). TcsL activated p38 MAP kinase as well, in line with recent observations from Hela cells (*24*). Combined treatment of J774A.1 cells with TcsL and TUDCA resulted in enhanced p38 MAP kinase activation compared to treatment with either TUDCA or TcsL alone (Figure 7A). p38 activation induced by either TcsL treatment

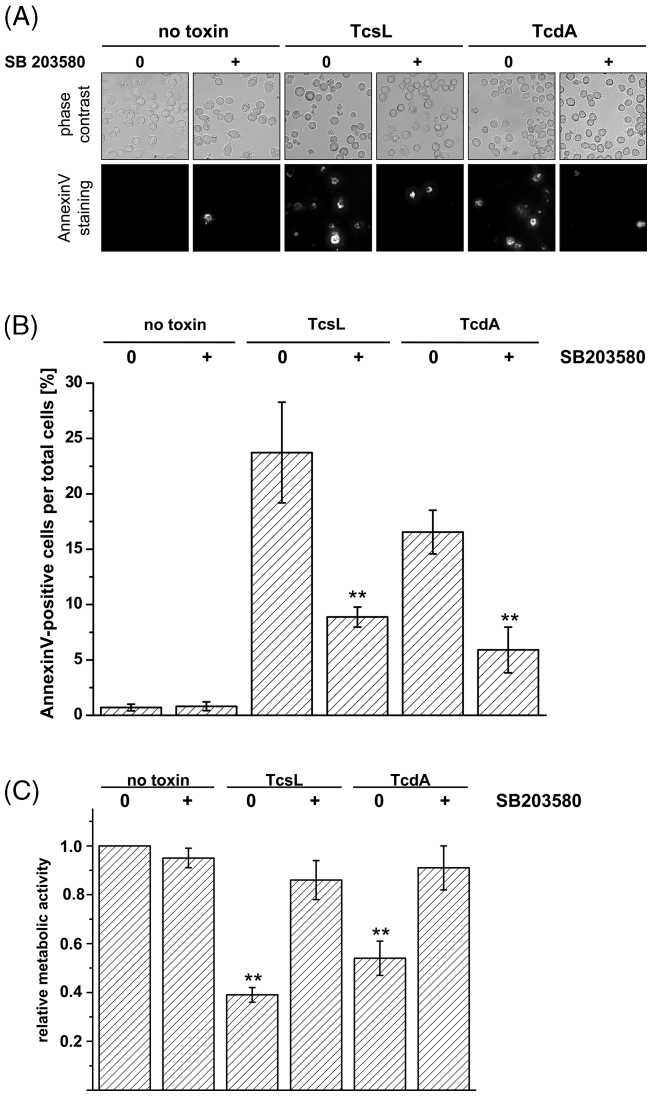

FIGURE 6: The p38 MAP kinase inhibitor SB203580 prevents toxin-induced apoptosis. J774A.1 cells either pretreated with SB203580 (10 μM) or not were exposed to TcdA or TcsL (100 ng/mL each) as indicated. (A) Phosphatidylserine exposure was analyzed by fluorescence microscopy after 12 h of toxin treatment. (B) Phosphatidylserine exposure was quantified as the ratio of annexin V-positive per total cells. (C) Cell viability was analyzed using the WST-1 test after toxin treatment for 24 h. Cell viability of nontreated cells was set to 1.0. Results displayed represent the mean of three independent experiments. *P*-values < 0.005 (**) were considered as statistically significant as compared with cells treated with either TcsL or TcdA alone.

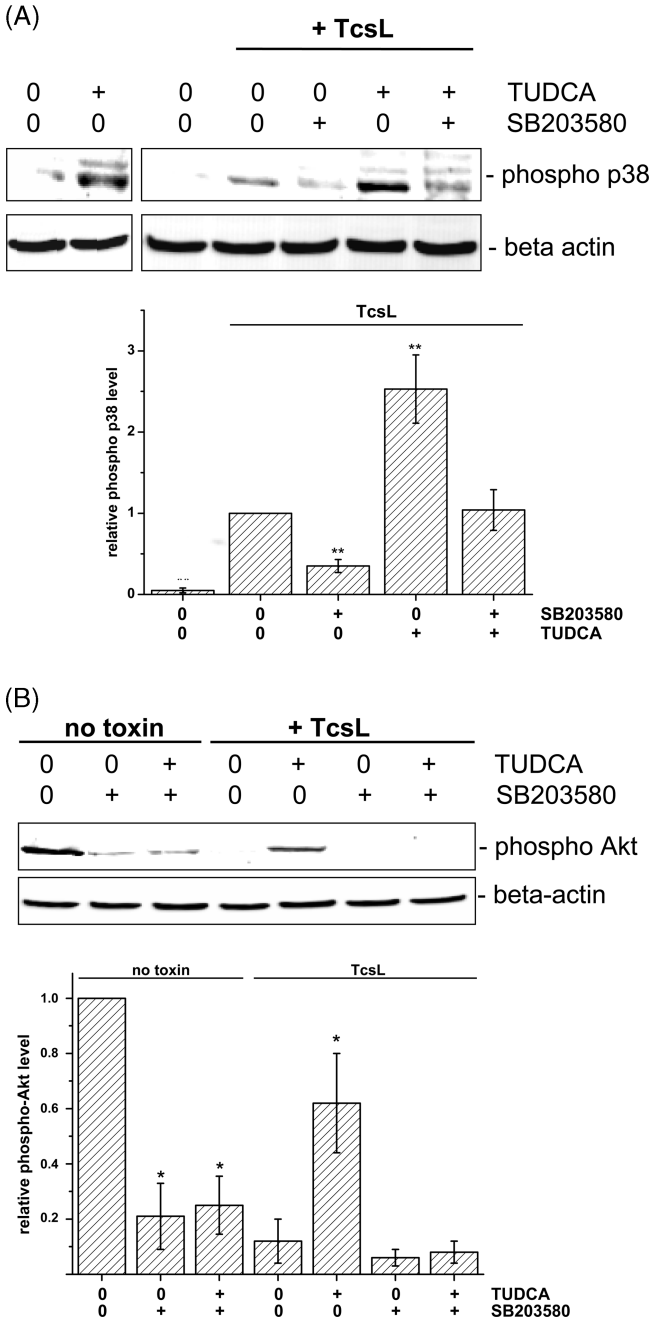

FIGURE 7: (A) TUDCA does not preserve TcsL-induced p38 MAPK activation. Serum-starved cells were pretreated with TUDCA (300 μM) or SB203580 (10 μM) and exposed to TcsL (100 ng/mL) for 6 h. Phosphorylation (indicative of activation) of p38 MAP kinase was analyzed by Western blotting. The phospho-p38 signal was densitometrically quantified and normalized to the β-actin signal. P38 phosphorylation of cells treated with TcsL alone was set to 1.0. Data shown represent the mean of three independent experiments. *P*-values < 0.005 (**) were considered as statistically significant as compared with TcsL-treated cells. (B) SB203580 does not preserve TcsL-induced Akt dephosphorylation. Cells were pretreated with TUDCA (300 μM) or SB203580 (10 μM) for 1 h. TcsL (100 ng/mL) was applied for 6 h, and the level of phospho-Akt was recorded by Western blot. The phospho-Akt signal was densitometrically quantified and normalized to the β-actin signal. Akt phosphorylation of nontreated cells was set to 1.0. Data shown represent the mean of three independent experiments. *P*-values < 0.05 (*) and < 0.005 (**) were considered as statistically significant as compared with either nontreated cells or TcsL-treated cells.

or by combined treatment with both TUDCA and TcsL was susceptible to inhibition by SB203580, an inhibitor of p38 MAP kinase (Figure 7A). Besides activating PI3K/Akt signaling, TUDCA thus activated p38 MAP kinase in TcsL as well as in untreated cells. This finding excluded that the antiapoptotic activity of TUDCA involved inhibition of p38 MAP kinase.

To investigate if the antiapoptotic activity of SB203580 involved preservation of Akt activity, J774A.1 cells were treated with SB203580 and analyzed for Akt phosphorylation. SB203580 induced Akt dephosphorylation (Figure 7B), in line with published data (*25*). In TcsL-treated J774A.1 cells, Akt was dephosphorylated regardless of the presence of SB203580 (Figure 7B). This finding excluded that the antiapoptotic activity of SB203580 involved preservation of Akt activity. These findings suggest that TUDCA and SB203580 exerted their antiapoptotic activity independently from each other through distinct signaling pathways. Combined treatment of J774A.1 cells with TUDCA and

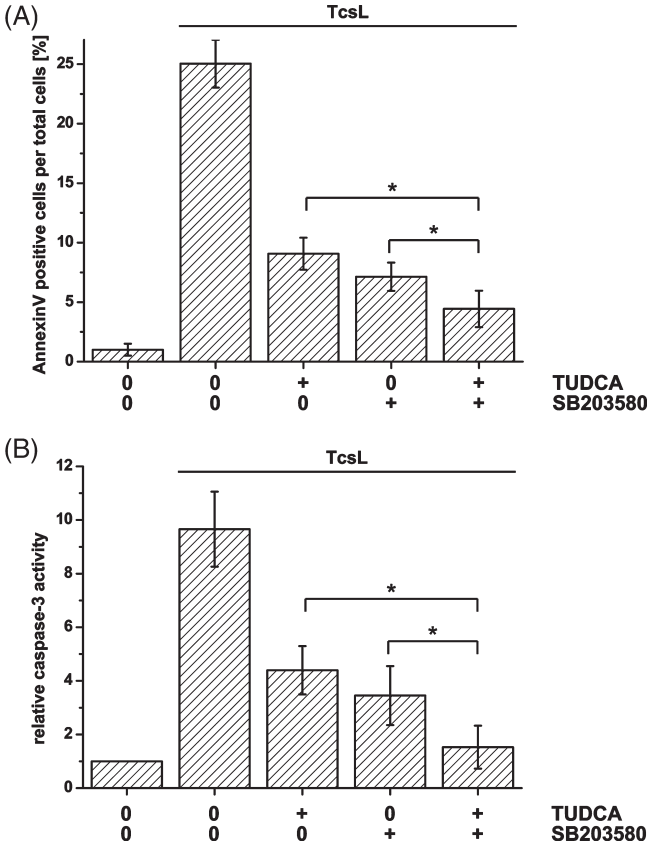

FIGURE 8: TUDCA and SB203580 protect independently from TcsL-induced apoptosis. (A) TcsL-induced phosphatidylserine exposure was analyzed in cells pretreated with TUDCA (300 μM) and SB203580 (10 μM) after toxin treatment for 12 h. The ratio of annexin V-positive per total cells was quantified. (B) The activity of caspase-3 was analyzed in cells pretreated with TUDCA (300 μM) and SB203580 (10 μM) after TcsL treatment for 6 h. Caspase-3 activity in nontreated cells was set to 1.0. Data shown represent the mean of three independent experiments. *P*-values < 0.05 (*) were considered as statistically significant.

SB203580 more efficiently prevented TcsL-induced phosphatidylserine exposure (Figure 8A) and caspase-3 activation (Figure 8B), compared to treatment with either drug alone.

DISCUSSION

Trophic factors trigger prosurvival pathways (including Ras/PI3K/PDK1/Akt signaling) and suppress proapoptotic pathways including p38 MAP kinase. Withdrawal of trophic factors results in a loss of prosurvival signaling and the desuppression of proapoptotic pathways (*26*) (Figure 9). Glucosylation of Rho/Ras proteins by glucosylating toxins is suggested to mimic the situation of the withdrawal of trophic factors, as they block trophic factor-induced signal transduction (e.g., Ras/Raf/ERK signaling) at the level of GTP-binding proteins (*27*−*29*) (Figure 9).

Against that background, we addressed the question whether reactivation of prosurvival signaling downstream of glucosylated (H/K/N)Ras is possible. To this end, we applied TUDCA known as an activator of PI3K/Akt signaling and provided evidence that TUDCA preserved PI3K/Akt signaling in TcsL-treated cells. Preservation of PI3K/Akt signaling by TUDCA turned out to be sufficient to prevent TcsL-induced apoptosis.

The high degree of similarity at the amino acid level among the clostridial glucosylating toxins has led to the hypothesis that

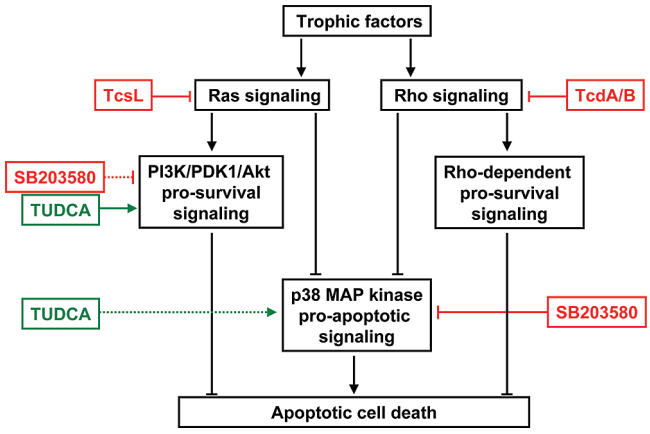

FIGURE 9: Pathways involved in TcsL- and TcdA/TcdB-induced apoptosis. The glucosylating toxins TcsL and TcdA/TcdB block trophic factor-induced signal transduction at the level of Ras/Rho proteins. The cell responds to the inhibition of Ras/Rho with activation of p38 MAP kinase. (H/K/N)Ras glucosylation by TcsL results in the inhibition of PI3K/PDK1/Akt survival signaling. TcsL-induced apoptosis is prevented if either PI3K/PDK1/Akt signaling is preserved by TUDCA or the activity of p38 MAP kinase is inhibited by SB203580. TcdA/TcdB-induced apoptosis is prevented if p38 MAP kinase is inhibited by SB203580. The TUDCA-induced activation of p38 MAP kinase does not contribute to the antiapoptotic activity of TUDCA. Furthermore, the SB203580-induced inhibition of PI3K/PDK1/Akt signaling does not contribute to the antiapoptotic activity of SB203580.

TcsL and TcdA/TcdB cause apoptosis by a comparable mechanism (*30*, *31*). We here provide evidence that TcsL and TcdA/TcdB differ in their responsiveness to TUDCA: TUDCA prevented TcsL-induced but not TcdA/TcdB-induced apoptosis, although TUDCA increased the level of phosphorylated Akt in TcdA/TcdB-treated cells as well (data not shown). The responsiveness to TUDCA correlated with the toxins' ability to glucosylate (H/K/N)Ras, the upstream activators of PI3K/Akt signaling. TUDCA prevented TcsL-induced apoptosis downstream of (H/K/N)Ras glucosylation, the apoptosis-inducing stimuli of TcsL (*10*), through preservation of PI3K/Akt signaling. In cells treated with TcdA/TcdB, (H/K/N)Ras is not glucosylated and PI3K/Akt signaling was obviously not critical for apoptosis. The Rho-dependent prosurvival signaling pathway that inhibition might be critical for TcdA-/TcdB-induced apoptosis is elusive. Based on correlation of TcdB isoforms with distinct proapoptotic activities and distinct substrate spectra, RhoA glucosylation (but not that of other Rho subtype proteins) is suggested to be critical for TcdB-induced apoptosis (*21*, *32*).

Apoptosis induced by the glucosylating toxins, however, shares common aspects as well: (i) TcsL as well as TcdA/TcdB induces expression of proapoptotic RhoB by inhibition of either Ras/PI3K/Akt signaling or a not yet identified RhoA-dependent signaling pathway, respectively (*32*, *33*). RhoB is suggested to be critical for TcdA/TcdB-induced apoptosis (*21*) and is most likely critical for TcsL-induced apoptosis as well (unpublished observation); (ii) inhibition of Rho/Ras-dependent survival signaling by either TcdA/TcdB or TcsL causes desuppression of proapoptotic signaling through p38 MAP kinase, which is required for TcdA/TcdB-induced apoptosis (*22*) (Figure 9). We here show for a first time that TcsL-induced apoptosis depends on p38 MAP kinase as well.

We further found that the p38 MAP kinase inhibitor SB203580 induced Akt dephosphorylation in nontreated cells. This effect is likely based on SB203580-induced inhibition of

phosphoinositide-dependent protein kinase 1 (PDK1), an upstream activator of Akt (*25*). Furthermore, TUDCA activated p38 MAP kinase, in line with published observations (*23*). These findings suggest that both TUDCA and SB203580 exhibit pleiotrophic effects. The TUDCA-induced activation of p38 MAP kinase, however, is not likely to contribute to the antiapoptotic activity of TUDCA, as p38 MAP kinase is already activated in TcsL-treated cells. The SB203580-induced Akt dephosphorylation is also not likely to contribute to the antiapoptotic activity of SB203580, as Akt is already dephosphorylated in TcsL-treated cells. In conclusion, (i) activation of PI3K/Akt (rather than activation of p38 MAP kinase) is critical for the antiapoptotic activity of TUDCA, and (ii) inhibition of p38 MAP kinase (rather than inhibition of PDK1/Akt) is critical for the antiapoptotic activity of SB203580.

Many diseases are attributed to unregulated apoptosis. An excess of apoptosis may result in T cell depletion, neurodegenerative diseases, or hepatocellular degeneration (*16*). Due to its antiapoptotic activity TUDCA is under investigation for its potential application as a therapeutic modulator of apoptosis-related diseases (*16*). In some cases, *C. sordellii* remains localized to the site of infection (*34*). TcsL-induced apoptotic cell death of phagocytotic cells may thereby represent a bacterial strategy to defend *C. sordellii* against eukaryotic phagocytosis (*10*). Application of TUDCA may help to prevent apoptotic cell death of phagocytotic cells (as well as of epithelial cells) and may subsequently better the clinical course of the diseases associated with toxigenic *C. sordellii*.

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REFERENCES

1. Aldape, M. J., Bryant, A. E., and Stevens, D. L. (2006) *Clostridium sordellii* infection: epidemiology, clinical findings, and current perspectives on diagnosis and treatment. *Clin. Infect. Dis. 43*, 1436–1446.
2. Voth, D. E., Martinez, O. V., and Ballard, J. D. (2006) Variations in lethal toxin and cholesterol-dependent cytolysin production correspond to differences in cytotoxicity among strains of *Clostridium sordellii*. *FEMS Microbiol. Lett. 259*, 295–302.
3. Kimura, A. C., Higa, J. I., Levin, R. M., Simpson, G., Vargas, Y., and Vugia, D. J. (2004) Outbreak of necrotizing fasciitis due to *Clostridium sordellii* among black-tar heroin users. *Clin. Infect. Dis. 38*, e87–e91.
4. Boehm, C., Gibert, M., Geny, B., Popoff, M. R., and Rodriguez, P. (2006) Modification of epithelial cell barrier permeability and intercellular junctions by *Clostridium sordellii* lethal toxins. *Cell Microbiol. 8*, 1070–1085.
5. Aktories, K., and Just, I. (2005) Clostridial Rho-inhibiting protein toxins. *Curr. Top. Microbiol. Immunol. 291*, 113–145.
6. Genth, H., Dreger, S. C., Huelsenbeck, J., and Just, I. (2008) *Clostridium difficile* toxins: More than mere inhibitors of Rho proteins. *Int. J. Biochem. Cell Biol. 40*, 592–597.
7. Reineke, J., Tenzer, S., Rupnik, M., Koschinski, A., Hasselmayer, O., Schrattenholz, A., Schild, H., and Von Eichel-Streiber, C. (2007) Autocatalytic cleavage of *Clostridium difficile* toxin B. *Nature 446*, 415–419.
8. Egerer, M., Giesemann, T., Jank, T., Satchell, K. J., and Aktories, K. (2007) Auto-catalytic cleavage of *Clostridium difficile* toxins A and B depends on cysteine protease activity. *J. Biol. Chem. 282*, 25314–25321.
9. Genth, H., Hofmann, F., Selzer, J., Rex, G., Aktories, K., and Just, I. (1996) Difference in protein substrate specificity between hemorrhagic toxin and lethal toxin from *Clostridium sordellii*. *Biochem. Biophys. Res. Commun. 229*, 370–374.
10. Dreger, S. C., Schulz, F., Huelsenbeck, J., Gerhard, R., Hofmann, F., Just, I., and Genth, H. (2009) Killing of rat basophilic leukemia cells by lethal toxin from *Clostridium sordellii*: critical role of phosphatidylinositide 3′-OH kinase/Akt signaling. *Biochemistry 48*, 1785–1792.
11. Boquet, P., and Lemichez, E. (2003) Bacterial virulence factors targeting Rho GTPases: parasitism or symbiosis ? *Trends Cell Biol. 13*, 238–246.
12. Halabi-Cabezon, I., Huelsenbeck, J., May, M., Ladwein, M., Rottner, K., Just, I., and Genth, H. (2008) Prevention of the cytopathic effect induced by *Clostridium difficile* toxin B by active Rac1. *FEBS Lett. 582*, 3751–3756.
13. Downward, J. (2004) PI 3-kinase, Akt and cell survival. *Semin. Cell Dev. Biol. 15*, 177–182.
14. Sola, S., Castro, R. E., Laires, P. A., Steer, C. J., and Rodrigues, C. M. (2003) Tauroursodeoxycholic acid prevents amyloid-beta peptide-induced neuronal death via a phosphatidylinositol 3-kinase-dependent signaling pathway. *Mol. Med. 9*, 226–234.
15. Beuers, U., Denk, G. U., Soroka, C. J., Wimmer, R., Rust, C., Paumgartner, G., and Boyer, J. L. (2003) Taurolithocholic acid exerts cholestatic effects via phosphatidylinositol 3-kinase-dependent mechanisms in perfused rat livers and rat hepatocyte couplets. *J. Biol. Chem. 278*, 17810–17818.
16. Sola, S., Aranha, M. M., Steer, C. J., and Rodrigues, C. M. (2007) Game and players: mitochondrial apoptosis and the therapeutic potential of ursodeoxycholic acid. *Curr. Issues Mol. Biol. 9*, 123–138.
17. Voth, D. E., and Ballard, J. D. (2007) Critical intermediate steps in *Clostridium sordellii* lethal toxin-induced apoptosis. *Biochem. Biophys. Res. Commun. 363*, 959–964.
18. Genth, H., Huelsenbeck, J., Hartmann, B., Hofmann, F., Just, I., and Gerhard, R. (2006) Cellular stability of Rho-GTPases glucosylated by *Clostridium difficile* toxin B. *FEBS Lett. 580*, 3565–3569.
19. Genth, H., Selzer, J., Busch, C., Dumbach, J., Hofmann, F., Aktories, K., and Just, I. (2000) New method to generate enzymatically deficient *Clostridium difficile* toxin B as an antigen for immunization. *Infect. Immun. 68*, 1094–1101.
20. Jiang, K., Sun, J., Cheng, J., Djeu, J. Y., Wei, S., and Sebti, S. (2004) Akt mediates Ras downregulation of RhoB, a suppressor of transformation, invasion, and metastasis. *Mol. Cell. Biol. 24*, 5565–5576.
21. Huelsenbeck, J., Dreger, S. C., Gerhard, R., Fritz, G., Just, I., and Genth, H. (2007) Upregulation of the immediate early gene product RhoB by exoenzyme C3 from *Clostridium limosum* and toxin B from *Clostridium difficile*. *Biochemistry 46*, 4923–4931.
22. Kim, H., Kokkotou, E., Na, X., Rhee, S. H., Moyer, M. P., Pothoulakis, C., and LaMont, J. T. (2005) Clostridium difficile toxin A-induced colonocyte apoptosis involves p53-dependent p21(WAF1/CIP1) induction via p38 mitogen-activated protein kinase. *Gastroenterology 129*, 1875–1888.
23. Schoemaker, M. H., Conde de la, R. L., Buist-Homan, M., Vrenken, T. E., Havinga, R., Poelstra, K., Haisma, H. J., Jansen, P. L., and Moshage, H. (2004) Tauroursodeoxycholic acid protects rat hepatocytes from bile acid-induced apoptosis via activation of survival pathways. *Hepatology 39*, 1563–1573.
24. Geny, B., and Popoff, M. R. (2009) Activation of a c-Jun-NH2-terminal kinase pathway by the lethal toxin from *Clostridium sordellii*, TcsL-82, occurs independently of the toxin intrinsic enzymatic activity and facilitates small GTPase glucosylation. *Cell Microbiol. 11*, 1102–1113.
25. Lali, F. V., Hunt, A. E., Turner, S. J., and Foxwell, B. M. (2000) The pyridinyl imidazole inhibitor SB203580 blocks phosphoinositide-dependent protein kinase activity, protein kinase B phosphorylation, and retinoblastoma hyperphosphorylation in interleukin-2-stimulated T cells independently of p38 mitogen-activated protein kinase. *J. Biol. Chem. 275*, 7395–7402.
26. Kummer, J. L., Rao, P. K., and Heidenreich, K. A. (1997) Apoptosis induced by withdrawal of trophic factors is mediated by p38 mitogen-activated protein kinase. *J. Biol. Chem. 272*, 20490–20494.
27. Herrmann, C., Ahmadian, M. R., Hofmann, F., and Just, I. (1998) Functional consequences of monoglucosylation of H-Ras at effector domain amino acid threonine-35. *J. Biol. Chem. 273*, 16134–16139.
28. Just, I., Selzer, J., Hofmann, F., Green, G. A., and Aktories, K. (1996) Inactivation of Ras by *Clostridium sordellii* lethal toxin-catalyzed glucosylation. *J. Biol. Chem. 271*, 10149–10153.
29. Welsh, C. F. (2004) Rho GTPases as key transducers of proliferative signals in G1 cell cycle regulation. *Breast Cancer Res. Treat. 84*, 33–42.
30. Petit, P., Bréard, J., Montalescot, V., El Hadj, N. B., Levade, T., Popoff, M., and Geny, B. (2003) Lethal toxin from *Clostridium sordellii* induces apoptotic cell death by disruption of mitochondrial homeostasis in HL-60 cells. *Cell. Microbiol. 5*, 761–771.
31. Matarrese, P., Falzano, L., Fabbri, A., Gambardella, L., Frank, C., Geny, B., Popoff, M. R., Malorni, W., and Fiorentini, C. (2007)

Clostridium difficile toxin B causes apoptosis in epithelial cells by thrilling mitochondria. Involvement of ATP-sensitive mitochondrial potassium channels. *J. Biol. Chem. 282*, 9029–9041.
32. Huelsenbeck, J., Dreger, S., Gerhard, R., Barth, H., Just, I., and Genth, H. (2007) Difference in the cytotoxic effects of toxin B from *Clostridium difficile* strain VPI 10463 and toxin B from variant *Clostridium difficile* strain 1470. *Infect. Immun. 75*, 801–809.
33. Gerhard, R., Tatge, H., Genth, H., Thum, T., Borlak, J., Fritz, G., and Just, I. (2005) *Clostridium difficile* toxin A induces expression of the stress-induced early gene product RhoB. *J. Biol. Chem. 280*, 1499–1505.
34. Sinave, C., Le, T. G., Blouin, D., Leveille, F., and Deland, E. (2002) Toxic shock syndrome due to *Clostridium sordellii*: a dramatic postpartum and postabortion disease. *Clin. Infect. Dis. 35*, 1441–1443.