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Bile salts increase epithelial cell proliferation through HuR-induced c-Myc expression

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Abstract

Background—Bile salts increase intestinal mucosal proliferation through an increase in c-Myc, a transcription factor that controls the expression of numerous translation regulatory proteins. HuR is an RNA-binding protein that regulates translation of target mRNAs. RNA-binding proteins can control mRNA stability by binding to AU- and U-rich elements located in the 3′-untranslated regions (3′-UTRs) of target mRNAs.

Aim—To determine how bile salt-induced c-Myc stimulates enterocyte proliferation.

Methods—Enterocyte proliferation was measured both *in vivo* using C57Bl6 mice and *in vitro* using IEC-6 cells after taurodeoxycholate (TDCA) supplementation. HuR and c-Myc protein expression was determined by immunoblot. c-Myc mRNA expression was determined by PCR. HuR expression was inhibited using specific small interfering RNA. HuR binding to c-Myc mRNA was determined by immunoprecipitation.

Results—TDCA increased enterocyte proliferation *in vivo* and *in vitro*. TDCA stimulates translocation of HuR from the nucleus to the cytoplasm. Cytoplasmic HuR regulates c-Myc translation by HuR binding to the 3′-UTR of c-Myc mRNA. Increased TDCA-induced c-Myc increases enterocyte proliferation.

Conclusions—Bile salts have beneficial effects on the intestinal epithelial mucosa, which are important in maintaining intestinal mucosal integrity and function. These data further support an important beneficial role of bile salts in regulation of mucosal growth and repair. Decreased enterocyte exposure to luminal bile salts, as occurs during critical illness, liver failure, starvation, and intestinal injury, may have a detrimental effect on mucosal integrity.

Keywords

Intestinal injury; Taurodeoxycholic acid (TDCA); c-Myc; HuR; Proliferation; Mucosal integrity

1. Introduction

Mucosal integrity is necessary for digestive and secretory functions as well as the formation of a barrier against noxious substances within the intestinal lumen. This integrity is maintained by a strict balance of proliferation, differentiation, and apoptosis [1,2].

Bile salts are normally found within the intestinal lumen, where their primary function is to aid in the absorption of lipids and lipid-soluble vitamins [3]. They also have cellular effects. The bile salt deoxycholic acid has been shown to increase proliferation and induce apoptosis in colon cancer cell lines [4,5]. Taurodeoxycholic acid (TDCA) increases growth of esophageal mucosa in a rabbit explant model [6]. The intestinal mucosa requires the presence of luminal contents to maximize the adaptive response [7-11]. After bile duct ligation, oral administration of sodium taurocholate improved anastomotic healing [12]. Animals with biliary diversion after small bowel resection demonstrate an impaired adaptive response [7-9]. Clearly, bile salts have many functions besides facilitating absorption of lipids and lipid-soluble vitamins. The presence of bile salts is important for regulation of these functions; their absence may be detrimental. Mucosal atrophy caused by starvation and critical illness, while multifactorial in etiology, may be related to cholestasis and diminished luminal bile. Further characterization of the beneficial functions of bile salts on the small intestinal mucosa is warranted.

Previous work from our lab has shown that TDCA supplementation into the growth media of intestinal epithelial cells results in increased cellular restitution and proliferation [13-15]. We have also shown that this increase in epithelial cell proliferation is due to a c-Myc-dependent pathway [16]. c-Myc is a proto-oncogene that plays a major role in the control of cell cycle [17,18]. Decreased expression of c-Myc mRNA has been associated with prevention of G₁-S phase transition and inhibition of cell proliferation [19,20]. The c-Myc gene codes for a nuclear phosphoprotein that functions as a transcription factor controlling cell division, differentiation, and apoptosis [17,18]. Constitutive expression of the c-Myc gene prevents exit from the cell cycle as well as differentiation [21,23]. Moreover, c-Myc activity is sufficient to drive resting cells into the cell cycle [22-24], and decreased expression of c-Myc gene prevents the transition from the G₁ to the S phase. c-Myc is a physiologic regulator of normal intestinal epithelial cell proliferation and is implicated in maintenance of mucosal epithelial integrity.

RNA-binding proteins like HuR control mRNA stability by binding to AU- and U-rich elements located in the 3'-untranslated regions (3'-UTRs) of target mRNAs [25,26]. HuR is a key regulator of genes that are central to cell proliferation, stress response, immune cell activation, carcinogenesis, and replicative senescence. HuR is predominantly nuclear in unstimulated cells, but when stimulated, HuR rapidly translocates to the cytoplasm, where it stabilizes specific mRNAs and affects the translation of target mRNAs [27,28]. We hypothesized that TDCA, which stimulates intestinal epithelial proliferation through a c-Myc-dependent pathway, increases c-Myc expression through an increase in HuR-regulated c-Myc expression. We studied the effect of TDCA on HuR binding to c-Myc mRNA and its effect on c-Myc expression.

2. Materials and methods

2.1. Animals

Six- to 8-wk-old C57Bl/6J male mice were used and were obtained from Jackson Laboratory (Bar Harbor, ME). Animals were housed in a standard facility, were kept on a 12-h light-dark cycle, and received water and mouse chow *ad libitum* until time for experimentation. All studies were approved by the University of Maryland School of Medicine Animal Studies Committee (IACUC protocol #0807008 and #1108007). Mice were treated in accordance with the National Institutes of Health laboratory animals use guidelines.

Mice underwent a sham laparotomy after isoflurane anesthesia. Postoperatively, the mice were placed on a liquid rodent diet (Micro-Stabilized Rodent Liquid Diet LD101; Purina Mills, St. Louis, MO) with or without the addition of TDCA (50 mg/kg/d TDCA, T0557;

Sigma-Aldrich, St. Louis, MO). Mice were sacrificed after 7 d of this treatment in order to evaluate intestinal proliferation.

2.2. *In vivo* quantification of intestinal proliferation

Quantification of intestinal proliferation was done in 100 well-oriented jejunal crypt-villus units in a masked manner. Mice were injected 90 min prior to sacrifice with 5-bromo-2'-deoxyuridine (BrdU) (200 μ L intraperitoneal injection per animal; Invitrogen Corporation, Carlsbad, CA) to label S-phase cells. BrdU was later detected via immunohistochemistry as previously described [29]. Briefly, paraffin-embedded slides were deparaffinized and rehydrated, and then underwent blocking of endogenous peroxidase activity. Slides were immersed in citric acid-based antigen unmasking solution (Vector, Burlingame, CA) and heated in a microwave for 15 min. After cooling to 42°C, sections underwent protein block (Dako, Carpinteria, CA) and were then incubated with rat monoclonal anti-BrdU (1:500; Accurate Chemical & Scientific) overnight at 4°C. On the subsequent day, slides were incubated with goat anti-rat secondary antibody (1:500; Accurate Chemical & Scientific) followed by incubation with streptavidin/horseradish peroxidase (Dako). Finally, sections were developed with diaminobenzidine and counterstained with hematoxylin.

2.3. Cell culture

The IEC-6 small intestinal cell line was purchased from the American Type Culture Collection at passage 13, and passages 15–20 were used for experimentation as previously described [16]. The cell line was derived from normal rat jejunal crypt cells and was developed and characterized by Quaroni *et al.* [30]. Dulbecco's modified Eagle medium supplemented with 5% heat-inactivated dialyzed fetal bovine serum, 1% gentamicin, and 0.1 U/mL insulin was used as control media. TDCA was supplemented to control media at 0, 0.05, 0.5, or 1.0 mmol/L when specified.

2.4. *In vitro* quantification of intestinal proliferation

IEC-6 cells were plated at 2×10^4 cells/cm² in Dulbecco's modified Eagle medium. After 24 h of growth, media were changed to contain 0 to 1 mmol/L TDCA ($n = 3$ –4 per group) as previously described [16]. Media were changed every 2 d thereafter and cells were harvested with 0.4% trypsin after 2, 4, or 6 d of growth. Total cell numbers were quantified using a hemocytometer and light microscopy and are reported as total cells per plate.

2.5. Flow cytometry

After 24 h of growth in varying doses of TDCA (0 to 1 mmol/L), 1×10^6 cells were resuspended in Tween buffer and incubated for 10 min at 37°C. Fetal bovine serum (FBS, 100 μ L; GIBCO, Grand Island, NY) was added and samples were spun for 7 min in a centrifuge at 500 *g*. The supernatant was removed and the pellet was resuspended in propidium iodide buffer as previously described [16]. After overnight incubation at 4°C, 300 μ L of PBS was added and the suspension was filtered through cell-strainer caps. To determine cell cycle phase, the samples were taken for flow cytometry analysis and all findings were confirmed in triplicate.

2.6. Western blot analysis

c-Myc and HuR protein expression was determined by Western blot analysis in growing IEC-6 cells exposed to TDCA-supplemented or control media. Protein extracts were dissolved in SDS sample buffer, heated to 100°C for 5 min, and subjected to electrophoresis on 10% acrylamide gels according to Laemmli [31]. Gels were then transferred to nitrocellulose membranes for 1 h and blocked with 5% nonfat dry milk in PBS–0.1% Tween

20 (PBS-T). The membranes were then incubated overnight at 4°C with either rabbit anti-c-Myc antibody (1:1000; Cell Signaling Technology, Danvers, MA) or mouse anti-HuR (1:1000; Santa Cruz Biotechnology, Santa Cruz, CA). The next day membranes were washed and incubated with secondary antibody (goat anti-rabbit IgG-HRP for c-Myc and goat anti-mouse IgG-HRP for HuR, 1:2000; Santa Cruz Biotechnology) for 1 h at room temperature. Membranes were developed and proteins detected using chemiluminescence reagents. EZQuant-Gel version 2.2 (EZQuant Ltd, Tel Aviv, Israel) was used to evaluate the density of protein expression of each membrane. Values are expressed relative to the density of protein expression grown in control media.

2.7. RNA isolation and PCR

After 24 h of growth in media supplemented with 0 or 0.5 mmol/L TDCA, IEC-6 cells were collected by centrifugation at 2000 *g* for 5 min as previously described [32]. Briefly, cells were lysed by trituration in 1 mL of TRIZOL reagent (Life Technologies) and then incubated at room temperature for 5 min. Chloroform (0.2 mL) was added and after 3 min of incubation the samples were spun in the centrifuge at 4°C for 15 min. An aqueous phase containing RNA (0.5 mL) was collected and isopropyl alcohol (0.5 mL) was added to precipitate RNA. This mixture was incubated for 10 min, respun in the centrifuge for 10 min, and washed with 1 mL of 75% ethanol. The RNA was finally dissolved in 400 µL of deionized water.

cDNA was reverse transcribed from 10 mg of total RNA using a first-strand cDNA synthesis kit (GIBCO BRL) and random hexamers [pd(N)₆ primer] as previously described [33]. The c-Myc PCR primer was derived from published sequences [34]. The specific sense and antisense primers for c-Myc included 5'-AATTTCTATCACCAGC-AACAG-3' and 3'-CCTCTACTACTGGCTCGATGA-5' and the expected size of the c-Myc fragment was 233 bp. For the PCR reaction, 2 mL of the first-strand cDNA reaction mixture was used and the cDNA samples were amplified in the thermal cycle under the following conditions: the mixture was annealed at 59°C (1 min), extended at 72°C (2 min), and denatured at 94°C (1 min) for 35 cycles. A final extension at 72°C (10 min) was done to ensure complete product extension. The PCR products were then electrophoresed through a 1% agarose gel and amplified cDNA bands were visualized by ethidium bromide staining. GAPDH was used as an internal control. All findings were confirmed in triplicate. The levels of β-actin PCR product were assessed to monitor the even RNA input in RT-qPCR samples. RT-qPCR was performed using 7500-Fast Real-Time PCR Systems (Applied Biosystems, Foster City, CA) with specific primers, probes, and software (Applied Biosystems).

2.8. RNA interference

Silencing RNA duplexes were designed to specifically cleave HuR mRNA and were synthesized and transfected into cells as described previously [29]. AACACGCT-GAACGGCTTGAGG was the sequence of small interfering RNA (siRNA) used that specifically targets the coding region of HuR mRNA (siHuR). The sequence of control siRNA (siCx) was AAGTG-TAGTAGATCACCAGGC and was synthesized and purchased from Santa Cruz Biotechnology. For each 60-mm cell culture dish, 15 µL of the 20 µM stock duplex siHuR or siCx was mixed with 300 µL Opti-MEM medium (Invitrogen) and this mixture was gently added to a solution containing 15 µL Lipofect-AMINE 2000 in 300 µL Opti-MEM. The solution was incubated at room temperature for 20 min and then gently overlaid onto monolayers of cells in 3 mL of medium. Cells were either quantified or harvested for various assays after 48 h of incubation.

2.9. Preparation of synthetic RNA transcripts

cDNA from IEC-6 cells was used as a template for PCR amplification of c-Myc at its coding region (CR) and 3'-UTR. The T7 RNA polymerase promoter sequence (T7) was contained in the 5'-primers: 5'-CCAAGCTTCTAATACG-ACTCACTATAGGGAGA-3'. Oligonucleotides (T7) 5'-TCTGCGAC-GAGGAAGAG-AAT-3' and 5'-TGCTCATCTGCTTGAACGGA-3' were used to prepare the CR of c-Myc (spanning position 537–1898). The c-Myc 3'-UTR template (spanning position 1899–2355) was prepared by using oligonucleotides (T7) 5'-ACTTACTGAGGAAACG-GCGA-3' and 5'-TAAGAGAAGGCTCAATTATATTT-3'. As described before, PCR-amplified products were used as templates to transcribe biotinylated RNAs by using T7 RNA polymerase in the presence of biotin-cytidine 5'-triphosphate [29].

2.10. RNA protein-binding assays

For biotin pulldown assays, biotinylated transcripts (6 µg) were incubated at room temperature with 120 µg of cytoplasmic lysate for 30 min. Complexes were analyzed by Western blot analysis after being isolated with paramagnetic streptavidin-conjugated Dynabeads (Dyna, Oslo, Norway). To assess the association of endogenous HuR with endogenous c-Myc mRNAs, immunoprecipitations (IP) of HuR-mRNA complexes were performed as previously described [29,35]. Briefly, 20 million IEC-6 cells were collected per sample and lysates were used for IP at room temperature for 4 h in the presence of excess IP antibody (30 µg; IgG or anti-HuR). RNA in IP materials was used in RT followed by PCR analysis to detect the presence of c-Myc mRNA.

2.11. Statistical analysis

A Student t-test was used when comparing two groups. When three or more groups were compared a 1-way analysis of variance with Newman-Keuls multiple comparison post-test was used. All data were analyzed using the Prism 5.0 statistical program (GraphPad Software, San Diego, CA) and are presented as mean ± SEM. Significance was considered with a *P* value of less than 0.05.

3. Results

3.1. TDCA supplementation increases proliferation

Exposure of IEC-6 cells to varying amounts of TDCA supplementation for 24 h resulted in a significant increase in the percentage of S-phase cells (20.7% ± 1.1% with no TDCA and up to 35.0% ± 0.5% with 1.0 mM/L TDCA, Fig. 1A) as determined by propidium iodide flow cytometry. This change in cell cycle phase translated into an effect of increased total cell number after 6 d of growth in TDCA-supplemented media. Cells were counted with a hemocytometer after staining with 0.4% trypan blue, and we saw a significant increase in total cell numbers (9.7 ± 1.0 × 10⁶ with no TDCA and up to 15.4 ± 0.8 × 10⁶ total cell count with 1.0 mM/L TDCA, Fig. 1B). All changes were also significant at a dose of 0.5 mM/L TDCA and this dose was used for all subsequent *in vitro* experiments.

Mice fed a liquid diet supplemented with TDCA at 50 mg/kg/d showed significantly increased cell proliferation. Crypt cell proliferation was quantified by counting S-phase cells in animals that were injected with BrdU 90 min prior to sacrifice (Fig. 1C–3D; *n* = 4–5). Animals whose diet was supplemented with TDCA had significantly increased numbers of cells undergoing proliferation when compared to animals whose diet was not supplemented (989 ± 94 versus 1186 ± 116 per 100 crypts; Fig. 1C). These data are the first to correlate our *in vitro* data to an animal model and show that TDCA supplementation *in vivo* causes increased crypt cell proliferation.

3.2. TDCA increases proliferation through a c-Myc-dependent pathway

To further evaluate what caused the increase in S-phase cells in the first 24 h, we chose to look at c-Myc expression after 24 h of TDCA supplementation. c-Myc signals cell proliferation by signaling cell division and replication. c-Myc expression, determined by Western blot analysis, is increased when cells are grown in TDCA-supplemented media for 24 h (Fig. 2A). PCR amplification from cells grown in TDCA-supplemented media also shows an increase in c-Myc mRNA when cells were grown in TDCA-supplemented media for 24 h (Fig. 2B). Together, these findings show that TDCA supplementation increases cell proliferation through a c-Myc-dependent pathway. Inhibition of c-Myc expression with antisense significantly decreases intestinal epithelial cell proliferation (Fig. 2C).

3.3. TDCA supplementation increases HuR cytoplasmic translocation

Cells grown in TDCA-supplemented media for 0, 2, 4, or 8 h showed progressively increasing expression of both total and cytoplasmic HuR protein with a correlating decrease in the nuclear HuR expression (Fig. 3A). There is a significant 2-fold increase in the active, cytoplasmic HuR protein expression after 8 h of TDCA supplementation when compared to cells grown in control media only (Fig. 3B). These findings indicate that TDCA signals an increase in HuR expression and cytoplasmic translocation.

3.4. HuR silencing abolishes TDCA-induced proliferation and the increase in expression of HuR and c-Myc

siRNA targeting HuR (siHuR) or control siRNA (siCx) was transfected into cells growing in either control media or TDCA-supplemented media and total cell number was quantified 2 d later. Again, we see a significant increase in the total number of cells with TDCA supplementation when compared to cells grown in control media ($146 \pm 0.4 \times 10^5$ versus $113 \pm 0.5 \times 10^5$ total cell count in control media, Fig. 4). When cells were transfected with siHuR, there was a significant drop in cell number regardless of what media they were grown in ($44 \pm 0.5 \times 10^5$ in control media and $69 \pm 0.8 \times 10^5$ in TDCA-supplemented media, Fig. 4). We also looked at cells that were supplemented with TDCA and received control siRNA transfection. There is a slight decrease in total cell number with siRNA transfection; however, the effect of siHuR is much more exaggerated. Therefore, the marked decrease in cell growth seen with siHuR transfection can be attributed to the effect of blocking HuR and not to the transfection process alone.

siHuR was able to reduce the expression of HuR in cells grown in control media. This effect was also seen in those cells grown in TDCA-supplemented media and was able to diminish the HuR protein expression to control levels. Diminished levels of HuR, as seen with the siHuR transfection, correlated directly to the level of c-Myc expressed in those cells (Fig. 5A). In fact, addition of TDCA to the control media resulted in a 2-fold increase in the expression of both HuR and c-Myc. With the addition of siHuR, the expression of these proteins returns to control levels (Fig. 5B and C). These data lead us to believe that TDCA induces proliferation through the c-Myc pathway via HuR stabilization.

3.5. HuR directly binds to the c-Myc 3'-UTR

Given that HuR expression correlates directly with c-Myc protein expression and that there are multiple predicted binding sites for HuR on the 3'-UTR of c-Myc mRNA (Fig. 6A), we sought to determine whether HuR and c-Myc directly interact. We also tested the hypothesis that this association would be increased in the presence of TDCA. We tested this in two ways. First, we used biotinylated transcripts spanning the c-Myc 3'-UTR in RNA pulldown assays using cell lysates from IEC-6 cells grown in either control or TDCA-supplemented media. Western blot analysis of the pulldown material shows that the c-Myc 3'-UTR

transcript associates readily with cytoplasmic HuR and the intensity of this binding increases in the presence of TDCA (Fig. 6B). This binding is specific as there was no binding of HuR to the transcripts corresponding to the coding region (CR) of c-Myc mRNA; there is also no binding of actin to c-Myc at either the 3'-UTR or CR (Fig. 6B).

Second, we examined the association of endogenous c-Myc mRNA with HuR in IEC-6 cells grown in either control or TDCA-supplemented media through IP of ribonucleoprotein (RNP) complexes. As seen with RT-PCR and real-time PCR analysis, the RNP complexes immunoprecipitated using anti-HuR antibody did contain endogenous c-Myc mRNA (Fig. 7A–C). This association was significantly increased 4-fold in cells when TDCA was supplemented to the media (Fig. 7B and C). Importantly, the c-Myc mRNA was not detected in IPs of nonspecific IgG (Fig. 7A). All together, these findings support the hypothesis that TDCA-induced cellular proliferation occurs through cytoplasmic HuR binding to the 3'-UTR of c-Myc mRNA.

4. Discussion

Mucosal integrity is maintained by a delicate balance between cell growth, or proliferation, and cell death, or apoptosis. The restoration and maintenance of mucosal integrity requires replacement of lost cells by epithelial cell renewal. Mucosal function depends on a regulated rate of division of proliferating cells in the crypts of the small intestine. Mucosal growth is affected by a plethora of agents whose presence is triggered by nutritional intake and luminal factors [36]. Factors affecting the balance between cell loss and regeneration rapidly alter the healthy mucosa. Injury may develop if factors that lead to cell loss are increased and/or factors promoting new cells are decreased. In response to injury or loss of intestine, altered gene regulation results in an increase in cell proliferation to replace lost cells and improve intestinal function.

Bile salts given *in vitro* have proven beneficial to mucosal integrity by protection from apoptosis and stimulation of proliferation [16,37]. We have again shown that TDCA supplementation in an *in vitro* model of epithelial cell growth results in an increase in S-phase cells and an increase in proliferation, and that this occurs through a c-Myc-dependent pathway. Importantly, we have also shown that these data can be extrapolated to an *in vivo* animal model. With the simple addition of TDCA to the diet of mice, we see a significant increase in the basal level of crypt proliferation. These are the first *in vivo* data to support our previous work. This model can be used for future studies of TDCA and its effects on the murine intestine.

The proto-oncogene c-Myc is associated with transition of cells into S-phase and increased cell proliferation [19,20]. We have shown that c-Myc protein and RNA is elevated after only 1 d of intestinal epithelial cell growth in TDCA. This increase in c-Myc expression results in an increase in cell proliferation. In order to more fully understand what is occurring at the cellular level in that first day, we chose to look at protein expression of the RNA-binding protein HuR after TDCA supplementation.

Our results indicate that bile salts increased cytoplasmic HuR specifically bound to the 3'-UTR of c-Myc mRNA. This increase in cytoplasmic HuR binding to the 3'-UTR of c-Myc mRNA is accompanied by an increase in total and cytoplasmic levels of HuR as well as a decrease in the level of nuclear HuR expression. HuR is ubiquitously expressed and growing evidence shows that the cytoplasmic translocation of HuR is most important when looking at its target effects of transcript stabilization and translation control [28,38]. Inhibition of HuR expression with small interfering RNA against HuR (siHuR) results in a significant inhibition of bile salt-induced enterocyte proliferation and c-Myc expression. These data suggest that TDCA-induced cytoplasmic HuR causes increased cell proliferation by binding

to the 3'-UTR of c-Myc, which results in an increase in c-Myc expression, allowing the increase in c-Myc to induce changes in the cell cycle, specifically by increasing the number of S-phase cells.

Our observations are also consistent with previous studies that demonstrate that HuR binds to AU-rich elements of labile mRNAs at its 3'UTR [32]. With the RNA pulldown, we show that HuR protein binds directly to c-Myc mRNA at its 3'-UTR and not at its coding region. This is confirmed by RNA immunoprecipitation, where we see c-Myc RNA forming a complex with anti-HuR antibody and not with nonspecific anti-IgG. Importantly, TDCA supplementation increases this binding in both experiments.

Traditionally, bile salts were thought to be injurious to the gastrointestinal mucosa. Bile salts are not physiologically present in the stomach and esophagus, where they have been linked to mucosal injury at these sites [5]. Bile salts' effects have been extensively studied in the colon but not in the small intestine. Bile salts cause injury within the colonic mucosa and are thought to be involved in the pathophysiology of colon cancer development through a variety of mechanisms including c-Myc [33,39-42] and epidermal growth factor [43]. Carcinoma in the small intestine is rare, while carcinoma is very common within the colonic mucosa. Thus bile salts may induce malignant degeneration in the colon, but not in the small intestine. It is not surprising that bile salts can be injurious to areas within the gastrointestinal system's mucosa where they have no crucial functions, e.g., the esophagus and stomach. Bile salts are present in the lumen of the small intestine and have very important physiologic functions there. Previous studies have shown that bile salts have beneficial effects in the small intestine, including stimulating restitution after injury [13-15] and increasing enterocyte resistance to apoptosis [15,44]. These data support our hypothesis that bile salts help maintain mucosal epithelial integrity through increased cell renewal through an HuR-dependent pathway.

In summary, we show here that the addition of bile salts, specifically TDCA, is beneficial to the intestinal epithelial mucosa by causing increased epithelial cell proliferation both *in vitro* and *in vivo*. This increase in proliferation seems to be at least partially due to an increase in HuR stabilization of c-Myc mRNA. This study further supports a physiologic role of bile salts within the intestinal mucosa distinct from its known role in lipid digestion. TDCA can affect cellular functions within the intestinal mucosa and can affect growth both *in vitro* and *in vivo*. Therefore, we conclude that diminished levels of bile salts, as seen in critical illness, liver failure, and starvation, could be detrimental to mucosal integrity. Further studies are needed to elucidate the role of bile salts in mucosal intestinal injury.

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Fig. 1.

TDCA supplementation increases proliferation. *In vitro* proliferation was measured in IEC-6 cells that were grown for 24 h. They were then placed in either control media or media supplemented with 0–1 mmol/L TDCA. (A) Percent of S-phase cells after 24 h of TDCA supplementation was determined by propidium iodide flow cytometry. (B) Total cell number was calculated after 2, 4, or 6 d of growth in TDCA-supplemented media using 0.4% trypan blue staining and a hemocytometer. (C, D) *In vivo* proliferation was measured by counting the S-phase cells, labeled brown using BrdU staining, in the intestine of mice that were fed a diet with or without TDCA supplementation. (E) Total number of BrdU-stained cells was quantified in 100 contiguous crypts and mice supplemented with TDCA had significantly increased levels of intestinal proliferation. *P values shown are in comparison to the control group. (Color version of figure is available online.)

Fig. 2.

TDCA proliferation occurs through c-Myc. (A) Western blot showing expression of c-Myc protein after 1 d of growth in control media as compared to 0.5 mmol/L TDCA-supplemented media with graphic display of relative protein levels of c-Myc. Actin was used as the control. (B) Expression of c-Myc RNA extracted from IEC-6 cells grown in control media as compared to 0.5 mmol/L TDCA-supplemented media for 24 h with graphic display of relative RNA levels of c-Myc. GAPDH was used for control. (C) Real-time PCR (RT-qPCR) 24 h after exposure to TDCA as a ratio *versus* control. (C.a) Effects of antisense on TDCA-induced IEC-6 cell proliferation: 1 μ m c-Myc or control antisense was added to the media for 6 d in the presence or absence of 0.5 mM TDCA. (C.b) Using the same experimental groups, cells were counted using 0.4% trypan blue staining and a

hemocytometer. Values are mean $\pm$ SE. * $P < 0.05$ *versus* control, ** $P < 0.05$ *versus* TDCA alone and TDCA + control antisense.

Fig. 3.

TDCA increases HuR expression. (A) Western blots showing total, cytoplasmic, and nuclear HuR protein expression are shown with increasing time of TDCA exposure in IEC-6 cells. Actin, tubulin, and lamin B are used as controls, respectively. (B) Densitometry shows significant changes in cytoplasmic HuR levels at 8 h of TDCA exposure when compared to control.

Fig. 4.

Effects of HuR silencing on cell proliferation *in vitro*. IEC-6 cells were grown in control (Cx) media for 24 h, then transfected with short interfering (si)RNA targeting the HuR mRNA coding region (siHuR) or control siRNA (siCx) for 6 h. Cells were then grown in control media or media supplemented with TDCA. Total cell number was calculated 2 d later using 0.4% trypan blue staining and a hemocytometer.

Fig. 5.

Effects of HuR silencing on the expression of TDCA-induced HuR and c-Myc expression. After IEC-6 cells were grown in control media for 24 h, they were transfected with siHuR. They were then grown in either control or TDCA-supplemented media for 42 h after transfection and total whole-cell lysate protein was then harvested (A). HuR (B) and c-Myc (C) expression are both significantly increased with TDCA supplementation and decreased with siHuR transfection.

Fig. 6. Binding of HuR to c-Myc mRNA. (A) Schematic representation of c-Myc mRNA and the potential binding sites for HuR in its 3'-UTR. HuR binding to 3'-UTR of c-Myc mRNA in IEC-6 cells that were grown in either control or TDCA-supplemented media. Cytoplasmic lysates (120 μ g) prepared from the two groups were incubated with biotinylated transcripts (6 μ g) of c-Myc mRNA at its 3'-UTR or coding region. (B) Representative immunoblots from the HuR and c-Myc pulldown materials are shown. Actin is used as the control and is appropriately negative, showing that there is no nonspecific RNA to protein binding. Densitometry of HuR binding to the 3' UTR of c-Myc from bolt in B.

Fig. 7.

Association of endogenous HuR to endogenous c-Myc mRNA. (A) Whole-cell lysates from cells grown in either control media or media supplemented with TDCA were used for immunoprecipitation (IP) in the presence of anti-HuR antibody or nonspecific IgG. RNA in the IP material was used in RT-PCR reactions to detect the presence of c-Myc mRNA. The resulting PCR products (~1300 bp) were visualized in agarose gels. (B) Densitometry of the RT-PCR results of c-Myc mRNA shows a 4-fold increase in association when cells are treated with TDCA *versus* control media. (C) Real-time PCR (RT-qPCR) of IP as described above 6 and 24 h after exposure to TDCA as a ratio *versus* control. IgG was used for control for nonspecific binding.