ER stress contributes to renal proximal tubule injury by increasing SREBP-2-mediated lipid accumulation and apoptotic cell death

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Lhoták Š, Sood S, Brimble E, Carlisle RE, Colgan SM, Mazzetti A, Dickhout JG, Ingram AJ, Austin RC. ER stress contributes to renal proximal tubule injury by increasing SREBP-2-mediated lipid accumulation and apoptotic cell death. Am J Physiol Renal Physiol 303: F266–F278, 2012. First published May 9, 2012; doi:10.1152/ajprenal.00482.2011.—Renal proximal tubule injury is induced by agents/conditions known to cause endoplasmic reticulum (ER) stress, including cyclosporine A (CsA), an immunosuppressant drug with nephrotoxic effects. However, the underlying mechanism by which ER stress contributes to proximal tubule cell injury is not well understood. In this study, we report lipid accumulation, sterol regulatory element-binding protein-2 (SREBP-2) expression, and ER stress in proximal tubules of kidneys from mice treated with the classic ER stressor tunicamycin (Tm) or in human renal biopsy specimens showing CsA-induced nephrotoxicity. Colocalization of ER stress markers [78-kDa glucose regulated protein (GRP78), CHOP] with SREBP-2 expression and lipid accumulation was prominent within the proximal tubule cells exposed to Tm or CsA. Prolonged ER stress resulted in increased apoptotic cell death of lipid-enriched proximal tubule cells with colocalization of GRP78, SREBP-2, and Ca\(^{2+}\)-independent phospholipase A\(_2\) (iPLA2\(_{A2}\)), an SREBP-2 inducible gene with proapoptotic characteristics. In cultured HK-2 human proximal tubule cells, CsA- and Tm-induced ER stress caused lipid accumulation and SREBP-2 activation. Furthermore, overexpression of SREBP-2 or activation of endogenous SREBP-2 in HK-2 cells stimulated apoptosis. Inhibition of SREBP-2 activation with the site-1-serine protease inhibitor AEBSF prevented ER stress-induced lipid accumulation and apoptosis. Overexpression of the ER-resident chaperone GRP78 attenuated ER stress and inhibited CsA-induced SREBP-2 expression and lipid accumulation. In summary, our findings suggest that ER stress-induced SREBP-2 activation contributes to renal proximal tubule cell injury by dysregulating lipid homeostasis.

Cyclosporine A; nephrotoxicity; apoptosis; endoplasmic reticulum stress; 78-kilodalton glucose-regulated protein; sterol regulatory element-binding protein-2

THE ENDOPLASMIC RETICULUM (ER) is the principal site for the folding and maturation of transmembrane, secretory, and ER-resident proteins (42). ER-resident chaperones, including the 78-kDa glucose regulated protein (GRP78), GRP94, and calnexin, assist in folding newly synthesized proteins and prevent the accumulation of misfolded proteins. Impairment in protein folding or the accumulation of misfolded proteins results in ER stress and activates the unfolded protein response (UPR), three signaling pathways that inhibit general translation, upregulate the expression of ER chaperones, and increase the degradation of misfolded proteins (3, 42). Recent evidence suggests that renal tubular damage caused by select drugs or toxins results in ER stress and UPR induction (5, 36). GRP78 was highly expressed in proximal tubules of puromycin aminonucleoside-treated proteinuric rats (20). Furthermore, induction of ER stress by high levels of intracellular albumin led to caspase-12-dependent apoptosis and contributed to interstitial fibrosis (31).

Cyclosporine A (CsA) has been shown to induce ER stress in cultured human proximal tubular cells (PTCs) and in rats (34, 35). Long-term CsA treatment in humans is associated with progressive renal dysfunction, characterized by interstitial fibrosis, tubular atrophy, and apoptotic cell death (16, 40, 46). Isometric vacuolization (IMV) of PTCs is a marker of acute CsA nephrotoxicity (1, 2) and has been shown to be a manifestation of dilated ER and the formation of lipid droplets (6, 30). Induction of ER stress with tunicamycin (Tm) leads to reversible nephrotoxicity marked by IMV of PTCs and features of acute tubular necrosis (ATN) in mice (59). However, the underlying mechanism by which ER stress causes renal injury and cell death is relatively unknown.

We have reported that ER stress and activation of the UPR induces lipid dysregulation (45) via activation of the lipogenic transcription factor sterol regulatory element-binding protein-2 (SREBP-2) (4). Inactive precursors of the SREBPs are localized in the ER through their interaction with SREBP-cleavage activating protein, Scap (14), and Insig (47, 48). Upon cellular sterol depletion, the SREBP/Scap complex is translocated to the Golgi, where the site-1-serine protease (S1P) and site-2-zinc metalloproteinase (S2P) release the active SREBP transcription factor. The active form triggers the expression of genes that encode enzymes in the cholesterol/triglyceride biosynthesis and uptake pathways (12). We have shown that SREBP-2 is activated during ER stress through the same pathway that is triggered by sterol depletion (4). It has also been demonstrated that various insults, including CsA, increase renal cortical cholesterol accumulation (52).

Recent studies have demonstrated that lipid accumulation due to increased SREBP-1 and/or -2 activation can cause lipotoxic cell death in pancreatic β-cells (15, 43) and alveolar type II cells (37). Furthermore, SREBP-1/-2 can contribute to apoptosis by modulating the expression of proapoptotic factors, including caspase 7 (11) and Ca\(^{2+}\)-independent phospholipase A\(_2\) (iPLA2\(_{A2}\)) (23, 38). Here, we show a direct correlation between ER stress, SREBP-2 expression, lipid accumulation, and apoptotic cell death in PTCs of kidneys from mice treated with Tm or in human renal biopsy specimens showing...
CsA-induced nephrotoxicity. Experiments in cultured HK-2 cells demonstrated that CsA-induced ER stress leads to increased SREBP-2 activation, resulting in lipid accumulation and apoptosis. Inhibition of ER stress by GRP78 overexpression or SREBP-2 activation by AEBSF treatment protected HK-2 cells from lipid accumulation and cell death. Taken together, these findings provide evidence that ER stress contributes to renal PTC injury through a mechanism involving SREBP-2-dependent lipotoxicity.

**METHODS AND MATERIALS**

*Tm treatments in mice.* Twelve C57BL/6J mice, 13 wk of age, were injected intraperitoneally (ip) with PBS alone or 500 ng/g Tm (Sigma, St. Louis, MO) and killed 24 or 72 h later. One-half of the mice was perfusion-fixed with neutral buffered formalin. The kidneys were removed, cut open longitudinally, and embedded in paraffin. Kidneys from the remaining mice were embedded in optimum cutting temperature compound and frozen in liquid nitrogen. The animal studies and protocols were approved by the McMaster University Animal Research Ethics Board.

*Human kidney biopsies.* Paraffin blocks of the most recent six consecutive human kidney biopsies from patients who had been pathologically interpreted as showing acute CsA-induced nephrotoxicity were retrieved from the archives of St. Joseph’s Hospital in Hamilton, with the approval of the Institutional Research Ethics Board.

*Cell culture.* The immortalized human proximal tubular cell line, HK2, was obtained from ATCC and cultured in keratinocyte media containing 0.05 mg/ml bovine pituitary extract and 5 ng/ml human recombinant epidermal growth factor. Cells were either fixed in 4% paraformaldehyde for immunofluorescence or lysed with 4× SDS-PAGE buffer plus 25 μg/ml of the proteasome inhibitor N-acetyl-leucinal-leucinal-norleucinal (ALLN; Calbiochem, Gibbstown, NJ) and a protease inhibitor cocktail (Roche, Mississauga, Canada).

Fig. 1. Vacuolization and lipid deposition in renal proximal tubules of C57BL/6J mice treated with tunicamycin (Tm). Kidneys were processed for histological analysis 24 or 72 h after ip injection with Tm. In paraffin sections, hematoxylin and eosin (H&E) staining revealed vacuoles in proximal tubular cells after 72 h (arrows in C) but not at 24 h (B). Oil red O (ORO) staining of cryosections revealed small lipid droplets at 24 h and larger lipid deposition in damaged tubules at 72 h (arrows in E and F, respectively). PBS-injected mice did not exhibit vacuolization (A) or lipid deposition (D). Tm induced the unfolded protein response (UPR) as revealed by immunohistochemistry for CHOP in the nuclei of proximal tubules at 24 and 72 h postinjection (H and I). No such staining was seen in the PBS-injected mice (G). Specificity of CHOP immunostaining was validated using CHOP−/− mice injected with Tm. At 24 h, strong staining was seen in wild-type (WT) mice (J) while there was no staining in CHOP−/− mice (K). Bar = 50 μm. G, glomerulus.
Immunohistochemistry. Kidney sections (4 μm thick) were deparaffinized and incubated in 0.5% H2O2 in methanol for 10 min to inhibit endogenous peroxidase activity. Sections were blocked and immunostained for SREBP-2 (sc-8151; Santa Cruz Biotechnology, Santa Cruz, CA) or iPLA2 (160507; Cayman Chemical, Ann Arbor, MI). Primary antibodies were detected using a biotinylated secondary antibody (Vector Laboratories, Burlington, ON) and streptavidin-peroxidase (Zymed, San Francisco, CA). For CHOP immunostaining, the sections were blocked, antigen heat-retrieved in Retrieve-all-2 solution (Cedarlane Laboratories, Burlington, ON) for 30 min, and incubated with an anti-CHOP antibody (sc-575; Santa Cruz).
followed by a secondary horseradish peroxidase (HRP) antibody (Dako, Burlington, Canada). Sections were developed in Nova Red peroxidase substrate (Vector Laboratories) and counterstained with hematoxylin. Images were taken with a Leitz Laborlux S microscope.

**Double immunofluorescence.** Cryosections were fixed in normal-buffered formalin for 30 min and blocked. Sections were immunostained for GRP78 (sc-1080; Santa Cruz), followed by CHOP (sc-575; Santa Cruz). Primary antibodies were detected with fluorescently labeled anti-rabbit Alexa 488 and anti-goat Alexa 594 diluted 1:200 (Invitrogen, Burlington, ON). Sections were observed with a Zeiss AXIO imager Z1 fluorescent microscope.

**ORO staining and lipid analysis.** Oil red O (ORO; Sigma) staining was used for observing intracellular lipid content (22). Frozen sections were fixed in formalin, rinsed in 1× PBS and 70% ethanol, and incubated in a saturated, filtered solution of ORO in 70% ethanol for 2 h. ORO is usually observed in brightfield microscopy. However, we took advantage of its fluorescence (594 nm excitation) and combined it with immunofluorescence. After ORO staining, sections were blocked and incubated with anti-CHOP antibody. Following treatment with an Alexa 488 secondary antibody, sections were examined with a Zeiss AXIO imager Z1 fluorescent microscope.

HK2 cells were cultured on cover slips, treated, fixed, and incubated with ORO for 2 h. Following 2 h incubation with 60% 2-propanol, the ORO-2-propanol solution was quantified by spectrophotometry at 510 nm and normalized to total protein.

**Immunoblot analysis.** Protein lysates were separated on SDS-polyacrylamide gels under reducing conditions and transferred to nitrocellulose membranes. Immunoblot analysis was performed using antibodies directed against SREBP-2 (557037; BD Biosciences, Mississauga, ON), GRP78 (610978; BD Biosciences), CHOP (sc-793 and sc-7351; Santa Cruz), or β-actin (A5441; Sigma). Primary antibodies were detected using HRP-conjugated secondary antibodies (Dako). Membranes were developed using the Renaissance Chemiluminescence Reagent kit (Perkin-Elmer, Waltham, MA).

Filipin staining. HK2 cells were treated for 24 h on cover slips, fixed, and incubated for 2 h in a freshly prepared Filipin III (Sigma) solution (~50 μg/ml). A grain of Filipin III was dissolved in 10 μl of dimethyl sulfoxide, diluted with 200 μl of PBS, and used immediately. Staining was visualized with a Zeiss AXIO imager Z1 fluorescent microscope.

**TdT-mediated dUTP nick end labeling analysis.** Subconfluent HK2 cells were transfected with either the sterol response element (SRE) reporter construct, pSRE-green fluorescent protein (GFP), that contained three replicates of the SRE sequences in its promoter linked to GFP (13), pCMV-SREBP2–468 (ATCC) which produces the mature form of SREBP-2, or p-EGFP-C1 (Clontech, Mississauga, ON) as a transfection control. Furthermore, cells were cotransfected with p-EGFP-C1 and pCMV-SREBP2–468 to utilize GFP as a marker of cells expressing the mature form of SREBP-2. Transient transfections were performed using the FuGENE 6 Transfection Reagent (Roche Diagnostics). Cells were fixed 24 h after transfection in 4% paraformaldehyde phosphate-buffered sa-

![Fig. 3. Correlation between sterol regulatory element-binding protein-2 (SREBP-2) expression and ER stress in human kidneys with cyclosporine A (CsA)-induced pathology. Representative H&E staining of a human kidney biopsy with acute CsA nephrotoxicity shows isometric vacuolization, previously shown to represent dilated ER (A–C). B and C show increased magnification of the same area delineated by the box in A. Bar = 100 μm (A and B) or 20 μm (C). Consecutive sections of human kidneys with CsA-induced pathology were immunostained for GRP78 (D and F) or SREBP-2 (E and G). GRP78 and SREBP-2 staining colocalized to the same tubules (arrows in D and E; Bar = 50 μm). F and G show high-power views of vacuolized tubules.](http://ajprenal.physiology.org/doi/abs/10.1152/ajprenal.00482.2011)
line and processed for staining with a TdT-mediated dUTP nick end labeling (TUNEL). In Situ Cell Death Detection Kit (Roche) as described previously (7). 4',6-Diamidino-2-phenylindole (100 ng/ml) staining was used as a counter stain to visualize nuclear DNA. Twelve microscopic fields were assessed for each transfection experiment. Only cells that were positive for transfection, as indicated by the expression of GFP, were analyzed. Thus, calculations of the percentage of apoptotic cells are the percentage of TUNEL-positive cells among those cells positively labeled for transfection with GFP.

In vivo, kidneys were obtained from mice treated with 500 ng/g Tm for 72 h and from human biopsies of patients with CsA-induced nephropathy. PBS-injected mice were used as a control. Paraffin sections were stained with TUNEL using the TACS2 TdT Apoptosis Detection kit (Trevigen, Gaithersburg, MD). Incorporated biotinylated nucleotides were visualized using streptavidin-peroxidase and Nova Red substrate. Nuclei were counterstained with hematoxylin.

Infection with recombinant adenovirus. Adenovirus (Ad) GRP78 encodes the complete open-reading frame of human GRP78 cDNA (44). Subconfluent HK2 cells were infected with AdGRP78 at 10–20 multiplicity of infection for 24 h and treated with Tg (Sigma) or CsA (Sigma). Adβ-gal infection was used as an adenovirus control.

Isolation of total RNA and quantitative RT-PCR. HK2 cells were pretreated for 2 h with AEBSF (0.3 mM) followed by treatment with U-18666A (2 μg/ml), Tg (200 nM), CsA (10 μg/ml), or Tm (2 μg/ml) for 24 h. Total RNA was isolated by using a RNeasy Mini Kit (Qiagen, Toronto, ON). A High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Burlington, ON) was used to transcribe 2 μg of RNA to cDNA. Real-time quantitative RT-PCR (qRT-PCR) analyses were performed with reverse and forward primers using the Power SYBR Green kit (Applied Biosystems). Relative quantification of each gene was calculated after normalization to β-actin.

The list of primers is as follows: human (h) SREBP-2: forward 5′-CCCTTCAGGACGTCATTACAC-3′, reverse 5′-TGCCATTGGCCGGTTGTGTC-3′; hLDLR: forward 5′-TGCTTCACTCCAAATCTCTCA-3′, reverse 5′-AAACCACTCTGAAAGACACT-3′; hHMGCoAR: forward 5′-TGATTTCCACCCGAAGGTCTT-3′, reverse 5′-GGGACACCTGTGCACTTA-3′; hβ-actin: forward 5′-ACCGAGCGCGGTACAG-3′, reverse 5′-CTTAATGTCAACGACAGTTC-3′.

Statistical analysis. Values are expressed as means ± SE. Comparison between the means of treatment groups were performed by Student’s unpaired t-test. Significance was recognized at the 95% level.

RESULTS

Tm induces proximal tubule lipid accumulation, ER stress, and SREBP-2 expression. To examine the physiological relevance of ER stress-induced SREBP-2 expression, we used a C57BL/6J mouse model of Tm-induced ER stress, through which intraperitoneal injection of Tm causes renal proximal tubule injury, resembling ATN. This model results in maximum serum creatinine, damage to proximal tubules, and weight loss at 3–4 days in mice. By 7–8 days, the animal has fully recovered (59). Consistent with this report, we observed

![Fig. 4. CsA causes ER stress and lipid accumulation. HK2 cells were treated with Tm (2 μg/ml), thapsigargin (Tg, 200 nM), or CsA (10 μg/ml) for 24 h. Total RNA was isolated by using a RNeasy Mini Kit (Qiagen, Toronto, ON). A High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Burlington, ON) was used to transcribe 2 μg of RNA to cDNA. Real-time quantitative RT-PCR (qRT-PCR) analyses were performed with reverse and forward primers using the Power SYBR Green kit (Applied Biosystems). Relative quantification of each gene was calculated after normalization to β-actin.](http://ajprenal.physiology.org/)
a decrease in body weight of 10% at 72 h postinjection. Proximal tubule cells of the kidney cortex showed vacuolized cells at 72 h (Fig. 1C), but not 24 h (Fig. 1B), postinjection. ORO staining of kidney cryosections revealed small lipid droplets in proximal tubules as early as 24 h (Fig. 1E) and larger lipid droplets in damaged tubules at 72 h (Fig. 1F). Distal tubules and glomeruli were not affected. No histological change or lipid staining was observed in PBS-injected mice (Fig. 1, A and D). The induction of ER stress was confirmed by immunohistochemistry for CHOP. Strong nuclear expression of CHOP was observed in proximal tubules at 24 and 72 h (Fig. 1, H and I) that was not observed in PBS-injected animals (Fig. 1G). As a control for anti-CHOP antibody specificity, nuclear staining for CHOP was absent in proximal tubule cells from Tm-treated CHOP−/− mice (Fig. 1K) compared with control C57BL/6J mice (Fig. 1J).

ER stress was seen in proximal tubules well before tubular damage was evident in hematoxylin and eosin (H&E) sections. At 24 h postinjection of Tm, ER stress markers GRP78 and CHOP colocalized to proximal tubules (Fig. 2A). GRP78 immunostaining was observed to be cytoplasmic, whereas CHOP immunostaining was nuclear. In control mice injected with PBS, only basal levels of GRP78 were observed with no CHOP immunostaining (Fig. 2A). Double-staining of cryosections for ORO and CHOP revealed small lipid droplets present in tubules of Tm-injected mice (Fig. 2B), but not PBS-injected mice. Tubules that contained lipid droplets also displayed intense nuclear immunostaining for CHOP (Fig. 2B), suggesting a functional relationship between ER stress and lipid accumulation.

Serial sections of paraffin-embedded kidneys from Tm-injected mice were immunostained for SREBP-2 or CHOP. Consistent with the increase in lipid droplets, increased SREBP-2 immunostaining was observed in the proximal tubules that exhibited strong nuclear staining for CHOP (Fig. 2C).

**ER stress markers and SREBP-2 colocalize in proximal tubules of CsA-treated patients.** IMV was apparent in H&E-stained specimens of six archived human renal biopsies previously pathologically interpreted as consistent with acute CsA nephrotoxicity (Fig. 3, A–C). GRP78 and SREBP-2 immunostaining was found to be prominent in vacuolized areas (Fig. 3, D–G). In these tubules, GRP78 showed typical perinuclear staining in the apical portion of the cells (Fig. 3F).

**Fig. 5.** ER stress induces free cholesterol accumulation. HK2 cells were either untreated, treated with Tm (2 μg/ml), Tg (200 nM), or CsA (10 μg/ml) for 24 h, and stained with filipin. Arrows indicate the accumulation of free cholesterol in the cell membrane of treated cells, which is indicative of SREBP-2 activation. U-18666A, an inhibitor of intracellular cholesterol trafficking, was used as a positive control. Free cholesterol accumulation can be observed in lysosomes (arrowheads). Bar = 20 μm.
CsA induces ER stress and lipid accumulation in proximal tubule cells. HK2 cells were treated with various concentrations of CsA (5, 10, or 20 μg/ml) or with the ER stress inducers Tm (2 μg/ml) or thapsigargin (Tg; 200 nM). GRP78 and CHOP expression was induced in treated cells (Fig. 4A). Time course analysis revealed that CsA induced GRP78 and CHOP expression at 18 h (Fig. 4B). CsA induces ER stress through a mechanism involving the release of ER Ca\(^{2+}\) (Dickhout and Austin, unpublished observation), similar to Tg (7). To determine if ER stress was also associated with changes in lipid content, ORO staining was performed on HK2 cells treated without or with Tg (200 nM), Tm (2 μg/ml), or CsA (10 μg/ml) (Fig. 4, C-F). All three ER stress-inducing agents caused a significant increase in lipid accumulation (Fig. 4G), and CsA induced a significant dose-dependent increase (Fig. 4H).

CsA and ER stress induce free cholesterol accumulation in the cellular membranes of proximal tubule cells. HK2 cells were treated with Tg (200 nM), CsA (10 μg/ml), or Tm (2 μg/ml) and stained with filipin to identify free cholesterol in the plasma membranes (Fig. 5). Free cholesterol accumulation was observed in the membranes of treated cells, indicative of SREBP-2 activation. As a positive control, treatment with U-18666A, which blocks cholesterol transport from late endosomes and lysosomes to the ER, resulted in depletion of ER membrane cholesterol and activation of SREBP-2 (21, 27).

Blocking SREBP-2 activation prevents CsA-induced lipid accumulation in proximal tubule cells. Both Tg and CsA induced SREBP-2 activation, as measured by the presence of the cleaved, mature form of SREBP-2 (Fig. 6A). Consistent with previous work (4), we showed that ER stress-induced SREBP-2 activation and GRP78 induction can be blocked by AEBSF (Fig. 6A), a serine protease inhibitor known to inhibit the protease activity of S1P (32). AEBSF was also found to significantly inhibit ER stress-induced lipid accumulation in Tg- and CsA-treated HK2 cells (Fig. 6B). To determine whether the ER stress-mediated cleaved form of SREBP-2 was functionally active, HK2 cells were transiently transfected with pSRE-GFP, an enhanced green fluorescent protein (eGFP) reporter construct controlled by the sterol regulatory element promoter. Treatment with ER stress agents (Tm, Tg, CsA) increased GFP expression in HK2 cells (Fig. 7A) and cell lysates (Fig. 7B). GFP expression was also increased in cells treated with U-18666A or transiently transfected with the mature form of SREBP-2.

**SREBP-2 expression induces apoptosis in proximal tubule cells.** To demonstrate the effect of SREBP-2-mediated gene expression on cell viability, HK2 cells were transiently transfected with pSRE-GFP or pCMV-SREBP2-468, the transcriptionally active fragment of SREBP-2. The pEGFP-C1 plasmid was used as a transfection control. Transient transfection with the pSRE-GFP construct allowed for the identification of HK2 cells where endogenous SREBP signaling was activated. Apoptosis, as measured by TUNEL staining, was induced significantly in pSRE-GFP transfected HK2 cells showing positive GFP expression (Fig. 8, B and D), indicating that the activation of endogenous SREBP signaling was associated with apoptotic cell death. This effect was not due to expression of GFP, since HK cells overexpressing eGFP showed very little apoptotic cell death (Fig. 8, A and D). To determine if the transcriptionally active form of SREBP-2 directly contributed to this apoptotic cell death, cotransfections with pSRE-GFP and pCMV-SREBP2-468 were performed. In cells where active SREBP-2 overexpression was achieved, as shown by the pSRE-GFP reporter (Fig. 8C), apoptosis was increased significantly over eGFP controls (Fig. 8E). Because the expression of GFP derived from the pSRE-GFP reporter construct does not distinguish between the activation of SREBP-1 or -2, AEBSF was used to inhibit endog-
enous SREBP activation to examine if expression of the mature form of SREBP-2 (driven by the pCMV-SREBP2-468 construct) alone would induce apoptosis. Cotransfection of HK2 cells with the pSRE-GFP and pCMV-SREBP2-468 constructs caused a significant increase in apoptosis with the inhibition of endogenous SREBP activation via AEBSF treatment (Fig. 8, F and H). Furthermore, cells expressing only the active form of SREBP-2 showed significantly greater apoptosis than HK2 cells with endogenous activation of SREBP-1 or -2 (Fig. 8, F and H). Given that a threshold of SREBP expression is required to activate the pSRE-GFP reporter construct, cells expressing SREBP-2 below the level of activation were likely excluded from the previous analyses, biasing results toward cells highly expressing SREBP-2. To determine the amount of apoptosis in all transfected cells, cotransfection with the pEGFP-C1 and the pCMV-SREBP2-468 constructs was performed. This resulted in a significant increase in apoptosis over pEGFP-C1 transfection alone (Fig. 8, G and I).

In vivo experiments showed that the 72-h Tm treatment increased the number of TUNEL-positive cells in lipid-enriched mouse PTCs (Fig. 9B) compared with 24 h Tm treat-
Fig. 8. Activation of SREBP-2 expression induces apoptosis. HK2 cells were transiently transfected for 24 h with enhanced GFP (eGFP) alone (A), pSRE-GFP (B), or pSRE-GFP combined with pCMV-SREBP2-468 (C). Cells were fixed and then stained with TdT-mediated dUTP nick end labeling (TUNEL, red) to identify transfected apoptotic cells and DAPI (blue) to identify cell nuclei. Programmed cell death was increased significantly in cells having endogenous, active SREBP (B and D) and by the overexpression of SREBP-2 (C and E). Pretreatment with AEBSF (to inhibit the endogenous expression of all SREBPs) and cotransfection with pSRE-GFP and the active form of SREBP-2 (F) still demonstrated a significant increase in apoptosis over cells showing the activation of all endogenous SREBPs (H). SREBP-2 cotransfected with eGFP (G) significantly increased programmed cell death compared with eGFP transfection (I). Twelve images were quantified for each treatment. *P < 0.05. **P < 0.01. Bar = 50 μm.

ment (Fig. 9A), indicative of apoptotic cell death. Tubular atrophy and TUNEL-positive cells were also observed in kidney biopsies of CsA-treated patients (Fig. 9C), a finding consistent with previous studies (16, 40, 46). Furthermore, the expression of iPLA2β, an SREBP-2 target gene with proapoptotic characteristics (23, 38), was observed in SREBP-2- and GRP78-positive PTCs from mice treated with Tm for 24 (Fig. 9, D–F) and 72 (Fig. 9, G–I) h.

Overexpression of GRP78 reduces CsA-induced SREBP expression and lipid accumulation in proximal tubule cells. Previous studies have demonstrated that GRP78 overexpression protects cells from ER stress-induced apoptosis and lipid
accumulation (8, 18, 45). HK2 cells were infected with GRP78-expressing adenovirus or a β-galactosidase (β-gal) control to determine if attenuating ER stress could prevent CsA-induced SREBP-2 expression and lipid accumulation. Significant GRP78 overexpression was achieved using this system (Fig. 10A). SREBP-2 induction, in response to either CsA or Tg treatment, was reduced markedly by AdGRP78 infection compared with cells infected with the Adβ-gal adenovirus (Fig. 10B). This resulted in a significant decrease in lipid accumulation in GRP78-overexpressing cells treated with CsA (Fig. 10C).

DISCUSSION

We have previously shown that ER stress leads to SREBP-2 activation, resulting in lipid synthesis and uptake (4, 45). Consistent with our findings, Kammoun et al. (18) demonstrated that GRP78 overexpression inhibits SREBP-1c/2 activation in hepatocytes and reduces hepatic steatosis in insulin-resistant obese mice. Mice injected with Tm displayed proteinuria, IMV, apoptosis, and proximal tubular ER stress (59). Several studies have reported a correlation between ER stress and CsA nephrotoxicity; increased expression of GRP78 was reported in kidney biopsies from CsA-treated patients by microarray analysis and qRT-PCR (26). It was shown in vivo and in vitro that ER stress is induced by CsA, and its alleviation prevents nephrotoxicity in PTCs (9, 34, 35). Consistent with these findings, protection from CsA nephrotoxicity by ER stress preconditioning through chaperone upregulation has been shown in several renal cell lines (36). Despite these findings, the relationship between ER stress, SREBP activation, lipid accumulation, and nephrotoxicity has not been explored.

We report here that, in the Tm mouse model of acute nephrotoxicity, there is proximal tubular lipid accumulation that colocalizes with increased GRP78, CHOP, and SREBP-2 expression. In biopsies of patients with acute CsA-induced nephrotoxicity, select tubules displayed loss of nuclei, cell necrosis, and extensive IMV, consistent with previous findings (16, 40, 46). Increased GRP78 and SREBP-2 expression was observed in vacuolized areas. Similar results were seen in vitro, where inhibition of SREBP-2 activation prevented lipid accumulation, and relief of ER stress by GRP78 overexpression attenuated SREBP-2 activation and suppressed lipid accumulation.

The PTCs of Tm-injected mice are sensitive to ER stress and develop a pathological lesion resembling ATN in humans (59). This is likely mediated by the PERK arm of the UPR, since a deficiency of CHOP or GADD34 provides nearly complete protection from lesion development (29). Consistent with these findings, Kimura and colleagues demonstrated the role of the UPR in protecting cells from toxicity-induced renal cell damage. A knock-in mouse model of mutant GRP78 inhibited ER stress-induced GRP78 upregulation and accelerated chronic tubulointerstitial injury when subjected to protein overload (20). Furthermore, the protective effect of ER chaperone upregulation during the UPR has been shown in multiple renal cell lines with various cytotoxic agents (36).
SREBP activation is correlated with apoptosis in a variety of causes PTC apoptosis. Previous studies have shown that overexpression of the active, mature form of SREBP-2 induces proximal tubule toxicity is associated with increased lipid accumulation. We have also shown that SREBP activation occurs before the first signs of apoptosis, indicating that SREBPs and the maintenance of membrane fluidity are important for the instigation and progression of apoptosis (13). Furthermore, putative SREBP-2 binding sites have been identified in the promoter regions of the CASP7 and CASP2 genes, encoding human caspase 7 and human caspase 2 (11, 28). It was shown that SREBP-2 can bind and positively regulate their expression (28). Recently, genome-wide analysis of SREBP-2 function revealed a number of potential target genes involved in autophagy (39). Our study is the first, to our knowledge, that provides a mechanistic link between ER stress, lipid accumulation, and SREBP activation in proximal tubule injury and apoptosis.

Fig. 10. Overexpression of GRP78 decreases SREBP-2 expression and lipid accumulation. HK2 cells were infected with 10 or 20 multiplicity of infection (moi) adenovirus encoding human GRP78 (AdGRP78) or β-galactosidase (Adβ-gal) control for 24 h (A). Immunoblot analysis showed that 10 or 20 moi of AdGRP78 treatment increased GRP78 expression compared with the Adβ-gal control. Treatment of cells for 24 h with Tg was used as a positive control for GRP78 induction. After the 24-h period of infection, cells were treated for 18 h with Tg (200 nM) or CsA (10 μg/ml) to assess the effect of GRP78 overexpression on SREBP-2 activation (B). AdGRP78-mediated overexpression of GRP78 reduced the levels of mSREBP-2 in response to Tg or CsA, compared with Adβ-gal-infected controls. This reduction in mSREBP-2 levels corresponded to a significant decrease in ORO staining induced by CsA (C). *P < 0.05 vs. β-gal control. **P < 0.05 vs. Adβ-gal + CsA-treated cells.

In the present study, we demonstrate in vivo that Tm-induced proximal tubule toxicity is associated with increased ER stress, SREBP-2 activation, and lipid/free cholesterol accumulation. Our in vitro findings suggest that PTCs undergoing ER stress have increased SREBP activation, leading to de novo lipid biosynthesis and accumulation. We have also shown that overexpression of the active, mature form of SREBP-2 causes PTC apoptosis. Previous studies have shown that SREBP activation is correlated with apoptosis in a variety of cell types, including pancreatic β-cells (15, 43) and alveolar type II cells (37). SREBPs can be cleaved by caspase 3, resulting in a fragment distinct from that cleaved by S1P and S2P (33). This fragment is functional and activates the expression of SREBP-2 target genes. SREBP-2 activation occurs before the first signs of apoptosis, indicating that SREBPs and the maintenance of membrane fluidity are important for the instigation and progression of apoptosis (13). Furthermore, putative SREBP-2 binding sites have been identified in the promoter regions of the CASP7 and CASP2 genes, encoding human caspase 7 and human caspase 2 (11, 28). It was shown that SREBP-2 can bind and positively regulate their expression (28). Recently, genome-wide analysis of SREBP-2 function revealed a number of potential target genes involved in autophagy (39). Our study is the first, to our knowledge, that provides a mechanistic link between ER stress, lipid accumulation, and SREBP activation in proximal tubule injury and apoptosis.

Zager and colleagues (17, 53–56, 58) have extensively studied lipid accumulation in PTCs in response to ischemia and cytotoxic compounds, both in vivo and in vitro (17, 53–56, 58). One study found that free iron-induced oxidative stress leads to free cholesterol depletion in PTCs, resulting in increased cytotoxicity; in this circumstance, further free cholesterol depletion via mevastatin increased cytotoxicity (57). However, in PTCs with normal cholesterol content, cholesterol ester load induced cell death, which was exacerbated by the ER stress inducer A-23187 (51). In macrophages, free cholesterol accumulation has been shown to induce apoptosis through a UPR-mediated mechanism (10, 19, 41, 49, 50). Furthermore, transgenic overexpression of SREBP-2 in pancreatic β-cells reduced islet area and exacerbated diabetes (15). Our results support the concept that SREBP-2 activation is cytotoxic in PTCs.

Although our findings suggest a lipotoxic effect of SREBP-2 in renal tubule cells, it is possible that other cell death signals are activated. Indeed, we observed increased expression of iPLA2β, an SREBP-2-inducible gene with proapoptotic characteristics (23, 38), in SREBP-2- and GRP78-positive PTCs from mice treated with Tm. Indeed, SREBP-1 activation can induce cell injury/apoptosis in pancreatic β-cells via an iPLA2-mediated mechanism that promotes ceramide generation via sphingomyelin hydrolysis (24, 25). Expression of malfolded proinsulin-2 in Akita β-cells induces ER stress and SREBP-1 cleavage/activation, thereby increasing iPLA2-mediated cell death (24). Recent studies have also identified an SRE in the iPLA2 gene and that binding of active SREBP-2 to this SRE leads to iPLA2 gene transcription (38). Based on these findings, SREBP-2 activation has the potential of modulating several cellular pathways that regulate apoptotic cell death. Studies are now underway to determine whether the expression levels of iPLA2 in renal tubule cells and kidney tissue exposed to CsA correlate with ER stress or SREBP-2 activation.

In summary, our in vitro findings confirm in vivo observations that CsA-induced renal tubular lipid accumulation is associated with ER stress and SREBP-2 activation. Future studies will allow us to determine whether other tubular nephrotoxins cause ER stress-induced SREBP-2 activation and lipid dysregulation and whether this can be prevented by inhibiting ER stress induction (i.e., 4-PBA, TUDCA) or SREBP-2 activation (f坐ostatin, betulin) in vivo.
GRANTS
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DISCLOSURES
The authors have nothing to disclose.

AUTHOR CONTRIBUTIONS

REFERENCES

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34. pallet n, Bouvier n, Bendjialalah a, rabant m, Florino JP, hertig a, legendre c, beaune p, thervet E, Anglicheau d. Cyclosporine-induced endoplasmic reticulum stress triggers tubular phenotypic changes and death. Am J Transplant 8: 2283–2296, 2008.

35. pallet n, rabant m, xu-dubois yc, lecorre d, mucchielli mh, imbnaud s, agier n, hertig a, thervet e, legendre c, beaune p, anglicheau d. Response of human renal tubular cells to cyclosorine and sirolimus: a toxicogenomic study. Toxicol Appl Pharmacol 229: 184–196, 2008.


