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Tauroursodeoxycholic acid (TUDCA) abolishes chronic high salt-induced renal injury and inflammation

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Abstract

Aim: Chronic high salt intake exaggerates renal injury and inflammation, especially with the loss of functional ET_B receptors. Tauroursodeoxycholic acid (TUDCA) is a chemical chaperone and bile salt that is approved for the treatment of hepatic diseases. Our aim was to determine whether TUDCA is reno-protective in a model of ET_B receptor deficiency with chronic high salt-induced renal injury and inflammation.

Methods: ET_B-deficient and transgenic control rats were placed on normal (0.8% NaCl) or high salt (8% NaCl) diet for 3 weeks, receiving TUDCA (400 mg/kg/day; i.p.) or vehicle. Histological and biochemical markers of kidney injury, renal cell death and renal inflammation were assessed.

Results: In ET_B-deficient rats, high salt diet significantly increased glomerular and proximal tubular histological injury, proteinuria, albuminuria, excretion of tubular injury markers KIM-1 and NGAL, renal cortical cell death and renal CD4⁺ T cell numbers. TUDCA treatment increased proximal tubule megalin expression as well as prevented high salt diet-induced glomerular and tubular damage in ET_B-deficient rats, as indicated by reduced kidney injury markers, decreased glomerular permeability and proximal tubule brush border restoration, as well as reduced renal inflammation. However, TUDCA had no significant effect on blood pressure.

Conclusions: TUDCA protects against the development of glomerular and proximal tubular damage, decreases renal cell death, and inflammation in the renal cortex in rats with ET_B receptor dysfunction on a chronic high salt diet. These results highlight the potential use of TUDCA as a preventive tool against chronic high salt induced renal damage.

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Conflict of interest

The authors declare no conflict of interest.

Keywords

high salt diet; ET_B receptors; renal injury; cell death; CD4⁺ T cells

Introduction

Endothelin-1 (ET-1) is an endogenous 21 amino acid peptide that acts through two G protein-coupled receptors (ET_A and ET_B receptors) that bind ET-1 with the same affinity. Over-activation of ET_A receptors promotes vasoconstriction and hypertension, renal inflammation, fibrosis and hypertrophy. ET_B receptors in the pulmonary endothelium act as clearance receptors to maintain low circulating ET-1 levels. In the renal vasculature, the majority of ET_B receptors are localized to the endothelial and smooth muscle cells of the efferent arteriole, whereas ET_A receptors are predominant in afferent arteriole vascular smooth muscle cells and podocytes. ET_B receptors are expressed by renal tubules, including proximal tubules, but especially in the renal collecting duct where they promote Na⁺ and water excretion by inhibiting tubular reabsorption *via* nitric oxide and prostaglandin production.¹ Loss of ET_B receptor function results in hypertension and increased susceptibility to renal injury.²

Immune cells are capable of responding to and producing ET-1³⁻⁶. In particular, *in vitro* studies suggest that pharmacological blockade of the ET_B receptor leads to increased T cell activation.³ Moreover, ET_B receptor expression appears to be decreased in T cells of patients with autoimmune diseases like systemic sclerosis,³ compared to healthy individuals.

The endothelin system is one of the major pathways involved in salt-sensitive hypertension and the associated renal injury.¹ Shindo et al.⁷ demonstrated that overexpression of the ET-1 gene (*Edn1*) in a mouse model leads to salt-dependent hypertension and proteinuria. Administration of ET_A specific blockers is effective in attenuating salt-sensitive hypertension and kidney damage in experimental animal models.⁸⁻¹⁰ Further, circulating levels of ET-1 are elevated in patients with salt-dependent hypertension.¹¹ High salt diet activates ET-1 production in both endothelial and collecting duct cells.^{12,13} Thus, in the absence of functional ET_B receptors, high salt induces exaggerated activation of ET_A receptors resulting in hypertension, renal injury and inflammation.^{2, 14} The ET_B-deficient rat model lacks functional ET_B receptors in all organs except neuronal tissues² and has been utilized by our laboratory and others to demonstrate that over-activation of ET_A receptors or loss of functional ET_B receptors facilitate the progression of renal injury, while functional ET_B receptors provide protection.¹⁴⁻¹⁷ Further reports have demonstrated oxidative stress-dependent dysfunction of ET_B receptors in models of hypertension.¹⁸ Thus, the use of the ET_B receptor-deficient rat model is a highly relevant model of hypertension and renal injury.

Tauroursodeoxycholic acid (TUDCA) is an endogenous hydrophilic bile salt that results from the chemical conjugation of the natural occurring ursodeoxycholic acid (UDCA) with the amino acid taurine in the liver.¹⁹ Both UDCA and TUDCA are classified as chemical chaperones with cytoprotective properties, mostly due to their abilities to reduce the formation of reactive oxygen species,²⁰ prevent mitochondrial dysfunction²¹ and decrease the cellular apoptotic threshold.^{22, 23} Circulating levels of UDCA/TUDCA in the

body are normally extremely low; however, hibernating animals such as the black bear exhibit high plasma UDCA/TUDCA levels that are thought to help reduce cell death during long periods of low nutrient intake.^{24, 25} TUDCA is FDA-approved for treatment of liver diseases such as cholestasis,^{26, 27} cirrhosis²⁸ and hepatitis.²⁹ TUDCA has protective effects against apoptosis and/or mitochondrial dysfunction in experimental models of neurological disorders such as Parkinson's,^{30–32} Huntington's³³ and Alzheimer's^{34–37} diseases, as well as a rat model of myocardial infarction.³⁸

TUDCA also appears to provide protection against the development of kidney disease. Compared with vehicle-treated animals, TUDCA treatment prior to renal ischemia/reperfusion results in lower blood urea nitrogen levels and decreased renal histological injury,³⁹ as well as diminished necrosis and apoptosis.⁴⁰ Short-term treatment with TUDCA was also recently shown to protect against folic acid nephropathy in a mouse model of the disease.⁴¹ More recent studies report beneficial effects of TUDCA treatment against aldosterone-infused renal injury⁴² and diabetic nephropathy.^{43, 44}

In this study, we hypothesized that TUDCA treatment would prevent the development of proteinuria, albuminuria, and renal glomerular and/or tubular injury in a model of salt-sensitive hypertension, the ET_B-deficient rat. We further examined whether TUDCA treatment would blunt renal apoptosis and inflammation in response to a high salt diet in the ET_B-deficient rat model. The results demonstrate that TUDCA treatment prevents the development of high salt-induced proteinuria, albuminuria, and renal glomerular and cortical tubular injury, as well as reducing renal cortical cell death and CD4⁺ T cell infiltration, in the absence of functional ET_B receptors and independent of lowering blood pressure.

Results

TUDCA treatment abolishes high salt-induced proteinuria and albuminuria

To assess if treatment with TUDCA protects against the development of salt-induced renal damage, ET_B-deficient rats and their transgenic (TG) control littermates were fed a diet containing either normal (0.8% NaCl) or high (8% NaCl) salt content for 3 weeks. Animals fed the high salt diet received daily i.p. injections of either TUDCA or vehicle. TG control rats fed normal and high salt diets had similar urinary excretion of protein and albumin at the end of the 3-wk protocol, and TUDCA did not significantly alter those values (Figure 1a). In contrast, ET_B-deficient rats fed the high salt diet developed kidney damage, as indicated by the significantly higher urinary excretion of protein and albumin compared to the same genotype fed the normal salt diet. TUDCA treatment significantly reduced urinary protein and albumin excretion by high salt-fed ET_B-deficient rats (Figure 1a), indicating a renoprotective effect of TUDCA under these conditions.

TUDCA treatment ameliorates excretion of KIM-1 and NGAL, but not nephrin

To further explore the specific nature of the high salt diet-induced kidney injury, urine collected at the end of the 3-wk protocol was assayed for markers of tubular damage, kidney injury molecule-1 (KIM-1) and neutrophil gelatinase-associated lipocalin (NGAL), and nephrin, a marker of podocyte damage. Similar to the increases seen in protein and albumin

excretion, ET_B-deficient rats fed a high salt diet displayed elevated urinary excretion of KIM-1 and NGAL compared to the same genotype fed a normal salt diet (Figure 1b), and this effect was attenuated by TUDCA treatment. KIM-1 content in renal cortex was also elevated in high salt-fed ET_B-deficient rats and this effect was markedly blunted by co-treatment with TUDCA (Figure 1b). Dietary salt intake and TUDCA treatment did not alter these markers of proximal tubular damage in TG control rats. Both genotypes responded to the high salt feeding with similar levels of nephrinuria, and TUDCA failed to ameliorate this effect (Figure 1b).

TUDCA upregulates megalin expression in the proximal tubule and decreases glomerular permeability to albumin

The proximal tubule has a key role in the reabsorption and processing of albumin filtered by the glomerulus^{45–47}, and studies have reported that disruption of this process results in albuminuria and proteinuria⁴⁸. To elucidate the effects of TUDCA on proximal tubule albumin reuptake mechanisms, immunohistochemical analysis was used to determine the expression of the endocytic receptor megalin. Although treatment with TUDCA did not result in any significant change in transgenic control rats, TUDCA led to a significantly elevated expression of megalin in proximal tubules of ET_B-deficient rats (Figure 2a). These findings suggest that TUDCA may increase the reabsorption of filtered albumin by the proximal tubule.

To investigate the effects of TUDCA on glomerular function, Wistar Kyoto rats were fed high salt diet and treated with the ET_B receptor antagonist A-192126 in drinking water for 3 weeks; rats also received daily i.p. injections of TUDCA or vehicle for the length of the high salt period. At the end of the 3 weeks, glomeruli were isolated and glomerular permeability to albumin (P_{alb}) was compared between the groups. Pharmacological blockade of the ET_B receptor during high salt consumption led to elevated P_{alb} in the vehicle-treated rats (Figure 2b), indicating dysfunction of the glomerular filtration barrier. On the other hand, TUDCA-treated rats had lower P_{alb} compared to vehicle-treated rats, suggesting that treatment with TUDCA minimizes the detrimental effects of high salt consumption on the glomerular filtration barrier.

TUDCA ameliorates the development of glomerular and proximal tubule damage, as well as renal cortical cell death

Renal histological assessment demonstrated that ET_B-deficient rats fed a high salt diet develop exaggerated glomerular damage compared to TG controls on a high salt diet, as denoted by the presence of increased glomerulosclerosis, cortical interstitial fibrosis and exaggerated thickening of the glomerular capsule (Figure 3). The glomerular injury was accompanied by an apparent loss of brush border integrity in the proximal tubules (Figure 4) and accumulation of iron in proximal tubule cells (Figure 5) in the ET_B-deficient rats. High salt feeding did not change the glomerular or proximal tubular morphology in TG control rats. On the other hand, treatment with TUDCA significantly prevented the development of glomerular injury, glomerular capsule thickening, brush border deterioration, cortical interstitial fibrosis and tubular iron deposition in the ET_B-deficient rats (Figures 3–5), while

not affecting the kidney morphology in TG control rats. The number of glomeruli and glomerular surface area were similar among the experimental groups (Table 1).

One of the major mechanisms through which TUDCA is reported to be protective against liver damage is by increasing the threshold for cell death.²¹ To determine if TUDCA protects against salt-induced kidney damage by decreasing cell death in the kidney, TUNEL-positive cells were counted in kidney sections from TG control and ET_B-deficient rats fed normal or high salt and treated with vehicle or TUDCA. As seen in Figure 6, TG control and ET_B-deficient rats presented with comparable numbers of TUNEL-positive cells when fed a normal salt diet, indicating similar instances of kidney cell death. In contrast, 3 weeks of high salt diet increased the number of TUNEL-positive cells (indicative of cell death) in the renal cortex of ET_B-deficient rats, while no significant changes were observed in the cortex of TG control rats fed high salt. Co-treatment with TUDCA during the high salt diet period prevented the salt-induced cell death in the cortex of ET_B-deficient rats, but did not induce any significant changes in the TG control rats (Figure 6). ET_B-deficient rats fed high salt and treated with vehicle or TUDCA displayed similar numbers of TUNEL⁺ cells in the outer medulla to ET_B-deficient rats fed normal salt diet (Figure 7). Interestingly, TUNEL⁺ cells in the cortex appear to be localized within the renal interstitium, and are non-tubular cells. The medullary TUNEL⁺ cells appear to be associated within the vasa recta bundles and vascular structures, although further in-depth analysis is needed to confirm the cell type(s) undergoing cell death in both regions.

TUDCA attenuates salt-induced CD4⁺ T lymphocyte accumulation in the renal cortex

Because renal inflammation is closely associated with kidney injury, the effects of high salt diet on renal immune cell numbers were quantified in ET_B-deficient and TG control rats. As seen in Figure 8a, high salt feeding induced greater T lymphocyte (CD3⁺ cells) infiltration in the renal cortex of ET_B-deficient rats than in TG control rats. Moreover, treatment with TUDCA significantly decreased T cell numbers, normalizing the numbers of T cells in the cortex to baseline levels. TUDCA significantly reduced outer medullary T lymphocyte numbers to similar levels in both genotypes on a high salt diet (Table 2). In contrast, salt-induced macrophage (ED-1⁺ cells) numbers in the cortex did not differ among genotypes or treatments (Figure 8b). TUDCA did not affect macrophage (ED-1⁺ cells) numbers in the renal outer medulla (Table 2) of high salt-fed rats. In the renal inner medulla, T-cell and macrophage infiltration were unaltered (Table 2).

To further characterize the inflammatory cells present in the renal cortex, flow cytometry was performed in cortical immune cells isolated from high salt-fed Wistar Kyoto rats treated with A-192126 and daily i.p. injections of TUDCA or vehicle for 3 weeks. The flow cytometry data demonstrate that the numbers of cortical leukocytes (CD45⁺ cells) did not differ between vehicle- and TUDCA-treated rats (Figure 9). However, TUDCA-treated animals presented with significantly smaller percentages of CD3⁺ cells in the cortex compared to animals treated with vehicle. Further, the effects of TUDCA on the cortical T lymphocyte population were specific to CD4⁺ cells, as the percentages of CD3⁺CD8⁺ cells did not differ between the experimental groups (Figure 9).

In concordance with the immunohistochemistry observations in high salt-fed ET_B-deficient rats, the flow cytometry results did not indicate an effect of TUDCA on the macrophage populations present in the renal cortex after three weeks of high salt feeding (% total events CD11b/c⁺ MHC-II⁺ cells, vehicle-treated vs. TUDCA-treated: 1.15 ± 0.3 vs. 0.85 ± 0.15 ; $P > 0.05$; $n = 5/\text{group}$).

TUDCA decreases TNF- α excretion, but does not change urinary oxidative stress markers

In response to high salt feeding, ET_B-deficient rats tended to increase urinary excretion of TNF- α , an indication of increased inflammation in the kidney. Further, treatment with TUDCA markedly suppressed TNF- α excretion by high salt-fed ET_B-deficient rats, without evoking any significant changes in TNF- α excretion in the TG control rats (Figure 10a). To investigate whether TUDCA may work by reducing renal oxidative stress, H₂O₂ and thiobarbituric reactive substances (TBARS) urinary excretion were assessed in TG control and ET_B-deficient rats after 3 weeks on the high salt diet and daily treatment with either TUDCA or vehicle. As shown in Figure 10b, urinary H₂O₂ excretion by TG control and ET_B-deficient rats did not differ significantly, and was unaltered by TUDCA. Similarly, there were no significant differences in urinary TBARS excretion (Figure 10c) across genotypes regardless of TUDCA treatment.

TUDCA treatment does not alter the renal ET system

To determine if treatment with TUDCA could be modifying the ET system in our animals, mRNA expression levels of pre-pro-ET-1 (*Edn1*), ET_A receptors (*Ednra*) and ET_B receptors (*Ednrb*) in renal cortex were determined by RT-PCR. As shown in Table 3, mRNA expression levels of these genes in renal cortex of rats fed high salt and treated with TUDCA were similar to those in rats fed high salt and treated with vehicle. Moreover, urinary excretion of ET-1, a marker of ET-1 production in the kidney, was unchanged by treatment with TUDCA in either genotype on high salt diet (TG control rats, vehicle vs. TUDCA: 17.9 ± 3.7 pg/day vs. 15.3 ± 2.2 pg/day; ET_B-deficient rats, vehicle vs. TUDCA: 18.0 ± 2.0 pg/day vs. 14.7 ± 2.0 pg/day; $P > 0.05$; $n = 5-6/\text{group}$).

TUDCA treatment does not decrease mean arterial pressure in high salt-fed ET_B-deficient rats

To determine if the protective effects of TUDCA against high salt-induced renal damage were due to decreases in blood pressure, 24-hr mean arterial pressure (MAP) was measured by telemetry in ET_B-deficient and TG control rats. Similar to previous observations,² ET_B-deficient rats fed a normal salt diet had elevated MAP when compared to TG controls (Figure 11). Upon initiation of the high salt diet, the MAP of ET_B-deficient rats further increased to a maximum of ~180 mmHg, but remained unchanged in the TG control rats. Treatment with TUDCA did not change MAP in either TG control or ET_B-deficient rats fed high salt diet. During the first week of high salt, no differences in MAP were observed between ET_B-deficient rats receiving vehicle and those receiving TUDCA; however, treatment with TUDCA significantly reduced MAP on days 9–12 of high salt feeding, although this difference in blood pressure between the groups was not sustained from day 13 to day 21.

TUDCA does not alter ER stress in the renal cortex

TUDCA is widely known as an inhibitor of the endoplasmic reticulum (ER) stress pathway.⁴⁹ To determine if TUDCA decreases salt-induced kidney damage by inhibiting ER stress, mRNA expression of ER stress markers (*GRP78*, *ATF-4*, *ATF-6*, *sXBP-1*, *CHOP* and *caspase-12*) was assessed in renal cortex of TG control and ET_B-deficient rats fed a high salt diet for 3 weeks and treated with TUDCA or vehicle for the same period of time. Table 3 shows that the expression levels of these markers of ER stress were similar across all groups. We conclude that TUDCA does not alter the development of salt-induced ER stress in the renal cortex.

Toll-like receptor pathways in the kidney are not altered by treatment with TUDCA

TUDCA has been reported to protect the liver against steatosis during transplantation by increasing expression of TLR4.⁵⁰ To determine if the renoprotective effects of TUDCA were due to changes in toll-like receptor pathways in this organ, *Tlr4* and *Tlr2* mRNA expression were assessed in renal cortex of TG control and ET_B-deficient rats after 3 weeks of high salt feeding and treatment with vehicle or TUDCA. Although ET_B-deficient rats tended to present slightly elevated expression of these genes when compared to high salt-fed TG control rats, statistical significance was not achieved (Table 3). Likewise, treatment with TUDCA did not significantly change the expression of these genes in renal cortex of ET_B-deficient rats (Table 3).

Discussion

The major findings of this study demonstrate that treatment with the endogenous hydrophilic bile salt TUDCA prevents the high salt-induced renal damage that is associated with ET_B receptor dysfunction, and that the renoprotective effects of TUDCA are independent of sustained blood pressure lowering. TUDCA also decreased high salt-induced cell death and T cell accumulation in the renal cortex of ET_B-deficient rats. In a model of pharmacological blockade of ET_B receptors, TUDCA explicitly lessened the cortical numbers of CD3⁺ CD4⁺ cells and ameliorated the glomerular filtration dysfunction associated with high salt consumption. Taken together, these results demonstrate that loss of ET_B function contributes to high-salt induced renal injury independent of hypertension, and that TUDCA or related therapeutics may be a potential approach for reducing the risk of chronic kidney injury.

Evidence in the literature demonstrates that ET_B receptors are reduced or dysfunctional in human hypertension,⁵¹ and that animal models of salt-sensitive hypertension like the Dahl rat also demonstrate dysfunction of this receptor.⁵² Using the ET_B-deficient rat as an animal model of salt-dependent renal damage,^{2, 14} and given the beneficial effects of TUDCA reported in the liver^{26–29} and other organs,^{30–33, 38} we tested if TUDCA would also be protective against the development of salt-induced renal damage. Recent studies demonstrated that treatment with TUDCA significantly decreases renal tubular injury in a mouse model of type 2 diabetes⁵³ and attenuates kidney damage mediated by increased aldosterone,⁴² suggesting a protective effect of this chemical chaperone in chronic kidney disease and specifically for renal tubules. We now provide evidence that TUDCA also

protects against the development of the kidney damage associated with ET_B receptor dysfunction after continued high salt intake.

Importantly, TUDCA treatment led to an elevated expression of megalin in the proximal tubules of high salt-fed ET_B deficient rats. Megalin is an endocytic receptor expressed in tubular epithelial cells, specifically in the brush border,^{54, 55} and it is involved in the proximal tubule reuptake of filtered proteins such as albumin.⁵⁵ A kidney-specific megalin knockout mouse exhibits albuminuria,^{56, 57} and biopsies obtained from patients of non-proteinuric renal diseases display elevated expression of megalin in proximal tubule when compared to patients of proteinuric diseases.⁵⁴ Surprisingly, TUDCA did not significantly diminish the urinary nephrin excretion nor did TUDCA normalize the glomerular permeability, since the P_{alb} was still about twice that in a normal rat,⁵⁸ supporting the notion that podocyte injury is still present, most likely due to the persistent hypertension. Thus, we propose that despite the remaining high salt-mediated podocyte damage in this model, proteinuria and albuminuria were ameliorated by a TUDCA-mediated compensation in the proximal tubule: upregulation of megalin in this segment of the nephron results in increased reabsorption of filtered protein and albumin, thereby, reducing excretion of protein and albumin and less subsequent injury to the tubules.

Prior studies have clearly demonstrated a role for ET-1 and ET_A receptor activity in contributing to glomerular injury in a variety of models such as diabetes and sickle cell disease.^{59, 60} Saleh et al.⁶¹ provided *in vivo* evidence that ET-1 directly increases glomerular permeability to albumin via the ET_A receptor, but that albuminuria is prevented by increased proximal tubule albumin uptake in the rat. Our results reveal that high salt induces proximal tubular cell damage in ET_B-deficient rats, suggesting that high salt-induced ET-1 may directly activate proximal tubular ET_B receptors to facilitate protein and albumin uptake. Further work is necessary to fully understand the role of the ET system in proximal tubular function and the albumin uptake mechanism.

Although reports in the literature indicate that TUDCA reduces inflammation in liver,⁶² intestine,⁶³ vasculature⁶⁴ and perhaps other organs, through downregulation of the ER stress response, TUDCA treatment in the present study did not decrease measures of ER stress in the cortex after high salt. This implies that the anti-inflammatory effects of TUDCA most likely are mediated by a different mechanism. For instance, it is possible that TUDCA could directly block the inflammasome pathway, which is involved in the development of several kidney diseases.^{65–67} Decreased renal expression of members of the NLRP3 inflammasome and decreased renal fibrosis have been described after treatment with TUDCA in a model of aldosterone-infusion kidney disease.⁴² Further studies are needed to test the possible effects of TUDCA on the renal inflammasome in the high salt fed ET_B-deficient rats.

Endogenous bile acids like TUDCA and UDCA reportedly modulate cell death by regulating death and survival transduction pathways in hepatocytes, neurons, lymphocytes and cardiomyocytes,^{21, 68, 69} among other cell types. TUDCA is thought to reduce the apoptotic threshold by modulating the glutamate-mediated activation of caspases and cytochrome c release.^{23, 70} We demonstrate that loss of ET_B function is associated with exaggerated high salt-mediated cell death in the renal cortex. TNF- α is one of the most potent inducers of cell

death⁷¹ and plays a role in the development of salt-sensitive kidney disease.^{72, 73} TUDCA significantly attenuated the excretion of TNF- α and also decreased cortical cell death in ET_B-deficient rats fed a high salt diet. Interestingly, TUDCA failed to decrease the numbers of TUNEL-positive cells in the outer medulla of ET_B-deficient rats. We speculate that TUDCA blunts TNF- α -dependent mechanisms that contribute to salt-mediated cortical cell death. Because TUDCA also decreased the CD4⁺ populations of T cells in the kidney of rats with ET_B dysfunction fed high salt, and CD4⁺ T cells produce TNF- α when activated,⁷⁴ TUDCA may be influencing TNF- α excretion through its inhibitory effects on the renal cortical CD4⁺ T cell populations. Our laboratory previously provided evidence that ET_A receptors contribute to renal T cell and macrophage accumulation in the angiotensin II-induced hypertensive mouse model.⁷⁵ Similar to the ET_B deficient rat, this model displays increased ET-1,⁷⁶ and down-regulation of renal ET_B receptors,⁷⁷ thus leading to over-activation of ET_A receptors. Further, ET_A selective blockade blunts angiotensin II-induced hypertension.^{75, 78–82} We also reported that T cell accumulation in the renal cortex of angiotensin II-induced hypertensive mice was mediated by ET_A receptors independent of blood pressure, while T cell accumulation in the medulla was blood pressure dependent.⁷⁵

We found similar numbers of TUNEL-positive cells in the outer medulla of ET_B-deficient rats regardless of salt content in the diet or TUDCA treatment. The outer medullary region is known to express ET_B receptors more abundantly than ET_A receptors.^{83, 84} Our group recently reported the critical role of ET_A receptor over-activation in promoting kidney cell death in a rat model of tunicamycin-induced acute kidney injury.⁸⁵ The increased numbers of TUNEL⁺ cells in the present studies indicate that the ET_B receptor opposes the actions of the ET_A receptor and, in the ET_B-deficient rats, where the ET_B receptor is dysfunctional, unopposed activation of the ET_A receptor leads to exaggerated cell death response especially in this area of the kidney. We propose that the mechanism of cell death in the renal medulla in this model of ET_B deficiency and high salt diet is distinct from that in the renal cortex and is hypertension-dependent.

ET_B-deficient rats fed a normal salt diet display elevated MAP when compared to TG controls, an effect attributed to elevated circulating ET-1 levels and ET_A activation.² Moreover, ET_B-deficient rats develop even further increases in MAP when fed a high salt diet, with further exaggerated circulating ET-1 levels.^{2, 14} Interestingly, the protective effects of TUDCA against kidney damage seen in the present studies are blood pressure-independent, as TUDCA did not cause a sustained decrease on MAP during the period of high salt intake. Loss of ET-1 or ET_B receptor in the nephron results in exaggerated salt-sensitive hypertension through negative effects on Na⁺ transport resulting in decreased natriuresis.^{86, 87} In addition, ET_B blockade in renal microvessels leads to exaggerated afferent arteriole vasoconstriction after high salt intake.⁸⁸ The unchanged arterial blood pressure that we observed in our experimental animals suggests that treatment with TUDCA does not affect Na⁺ transport or afferent arteriole sensitivity. Similarly, the protective effects of TUDCA cannot be attributed to modifications of the ET system given that the cortical expression of pre-pro-ET-1, ET_A and ET_B did not differ among genotypes or treatments. Furthermore, urinary ET-1 excretion, a well-known marker of renal ET-1 production, was similarly unaltered by TUDCA treatment. In addition, we found that ER stress and urinary oxidative stress-related parameters were unaltered by TUDCA in this model. However, we

recognize that the ER stress and oxidative stress measurements were limited and not performed at the cellular level; hence, further analysis is required to monitor ER stress and oxidative stress in isolated proximal tubules and/or T cells with ET_B deficiency under high salt conditions.

Perspectives

Western diets, especially in the United States, contain substantial amounts of salt. Many cardiovascular and renal diseases, especially with high fat diets and hypertension, lead to a loss of ET_B receptor function, which increases risk of salt dependent complications. ET_B receptors are reduced or dysfunctional in human hypertension⁵¹ and animal models of salt-sensitive hypertension like the Dahl rat.⁵² ET_B receptor deficiency and chronic high salt diet results in glomerular and proximal tubular injury, increased proteinuria and albuminuria, exaggerated renal cortical cell death, increased renal cortical T cell accumulation, and salt-sensitive hypertension. The loss of functional ET_B receptors may “prime” renal cells for high salt-induced injury possibly via over-activation of ET_A receptors. These studies indicate that high salt plus ET_B dysfunction promotes glomerular and proximal tubular injury leading to exaggerated albuminuria. This increase in albumin excretion with higher interstitial sodium may alone provide the stimulus for renal interstitial cell death and/or T cell activation. However, we cannot exclude the possibility that loss of ET_B receptors on interstitial cells and/or T cells promotes cell death and activation, respectively.

The chemical chaperone bile salt, TUDCA, may be a valuable therapeutic tool for chronic cardiovascular and renal diseases, which are exacerbated with high salt diets. TUDCA treatment abolished the proteinuria, albuminuria, and glomerular and cortical tubular injury but not the podocyte injury in the salt-induced ET_B-deficient rat model. Further, TUDCA treatment ameliorated the renal cortical cell death and T cell accumulation observed in the ET_B-deficient rat model independent of sustained changes in the salt-induced hypertension.

Materials and methods

Animal studies

All protocols were conducted in accordance with the *Guide for the Care and Use of Laboratory Animals*, and were approved by the Institutional Animal Care and Use Committee of the University of Alabama at Birmingham. Ten-to-twelve week old male ET_B-deficient rats (specifically, *DBH-ET_B;ET_B^{sl/sl}* rats) and their *DBH-ET_B;ET_B^{+/+}* transgenic littermates (TG control rats) were used in these studies. Both ET_B-deficient rats and TG control rats carry the transgene for the ET_B receptor driven by the dopamine β-hydroxylase promoter. This promoter rescues ET_B-deficient rats from a lethal phenotype by expressing a functional ET_B receptor in adrenergic tissues;² however, ET_B-deficient rats lack functional ET_B receptors in nonadrenergic tissues such as vascular endothelium and renal tubular epithelium. All animals were housed under constant temperature and humidity conditions, in a 12-h dark:12-h light cycle, with food and water available *ad libitum*.

Rats were randomly assigned to stay on either a normal salt diet (0.8% NaCl; LabDiet 5L3Z,) or a high salt diet (8% NaCl; Teklad 8604) for 3 weeks. The group of rats receiving

high salt diet was further divided in 2 subgroups receiving intraperitoneal injections of tauroursodeoxycholic acid (TUDCA, 400 mg/kg/day; EMD Millipore, Billerica, MA) or vehicle (isotonic saline) daily throughout the 3-wk period. Urine was collected on the last day of the study (after one day acclimatization to metabolic cages), a plasma sample was obtained, the animals were sacrificed, and the kidneys were harvested. In some cases, kidneys were divided into renal cortex and outer medulla, which were snap frozen in liquid nitrogen and kept at -80°C until further analysis. Other kidneys were fixed overnight in formalin and preserved in paraffin for immunohistochemistry studies. Because preliminary studies revealed no changes in the outer medulla, the present studies focused on the renal cortex. In a second set of animals, PA-C40 transmitters (Data Sciences International, Duluth, MN) were implanted in the abdominal aorta to allow monitoring of blood pressure in the conscious state, as previously described.¹² In brief, rats were anesthetized with 2–3% isoflurane, the abdominal aorta exposed and the telemeter implanted and held in place with tissue glue (Vetbond, 3M, St. Paul, MN). Animals recovered from surgery for one week prior to the beginning of the 3-wk protocol detailed above, during which mean arterial pressure (MAP) was recorded at 10-sec intervals every 10 min throughout the protocol.

In a second set of experiments, ten week-old Wistar Kyoto male rats received 8% NaCl diet and the selective ET_B receptor antagonist A-192621 (10mg/Kg/day in the drinking water, AbbVie Inc., North Chicago, IL) for 3 weeks, and were randomly assigned to receive daily i.p. injections of vehicle or TUDCA for the entirety of the high salt diet period.

Quantitative RT-PCR

RNA was extracted from renal cortex using RNeasy mini kit (Qiagen, Valencia, CA) and quantified by spectrophotometric analysis (NanoDrop ND-1000, Thermo Scientific, Waltham, MA). RNA reverse transcription was performed using Quantitect Reverse Transcription kit (Qiagen) following manufacturer's instructions. Primers for components of the ET system were purchased from QuantiTect (Qiagen; *Edn1*: QT00371308; *Ednra*: QT00182546 and *Ednrb*: QT01084454) and GAPDH was used as housekeeping gene (Qiagen, QT01658692). ER stress and toll-like receptor (TLR) primers were synthesized by Integrated DNA Technologies (IDT, Coralville, IA; primer sequences are indicated in Table 4). RNA expression was detected with Quantitect SYBR green kit (Qiagen) and using a CFX96 Touch RT-PCR detection system (Bio-Rad, Hercules, CA).

Histological assessment of renal damage

Gomori's Blue Trichrome was used to visualize renal structures using bright-field microscopy (Olympus BX40; Olympus America, Melville, NY). Photographs were obtained with a digital camera (Olympus DP12; Olympus America). In a blinded manner, 20 glomeruli per slide were examined and given a glomerulosclerosis score of 0 = 0%, 1 = 25%, 2 = 50%, 3 = 75%, or 4 = 100%. The 20 glomerulosclerosis scores per kidney were then averaged. Presence of interstitial fibrosis was also scored in blue trichrome-stained kidney slices, using a scale from 0 to 3, with 0 = no fibrosis present and 3 = extensive fibrosis.

Thickness of the glomerular capsule was evaluated by using picrosirius red staining. 20 glomeruli per animal were scored in a blinded manner using a scale of 1 = normal, 2 = moderately thick, 3 = very thick and 4 = extremely thick. The data is reported as the average score per experimental group.

For examination of proximal tubule brush border integrity, periodic acid-Schiff (PASH) stain was utilized. 20 fields per kidney were examined, each receiving a score for integrity (0 = no brush border, 1 = poor integrity, 2 = moderate integrity, and 3 = excellent integrity). The scores of the 20 fields/kidney were averaged and reported.

To evaluate possible differences in the number of glomeruli and glomerular surface area among the groups, a total of 20 microscopy fields were assessed per animal in hematoxylin and eosin (H&E)-stained kidney slices. The number of glomeruli per field was recorded and the average per animal was calculated. The glomerular surface area was measured for all whole glomeruli within a microscopy field using CellSens software (Olympus America).

Iron deposition in the renal cortex was assessed using standard Prussian blue histology staining protocol.⁵⁹

Immunohistochemical analysis

Kidneys were fixed overnight in 4% buffered formalin solution at room temperature, transferred to 70% ethanol for 24 h, and paraffin-embedded. Tissues were cut longitudinally into 4 μm -thick sections and mounted on Superfrost slides. Tissue sections were stained with primary antibodies specific for CD3 (1:400; Abcam, Cambridge, MA), ED-1 (1:100; Bio-Rad, Hercules, CA), and megalin (1:50,000; Abcam) and detected with polymer conjugated secondary antibody (Biocare Medical, Concord, CA). Quantification of renal T-lymphocyte (CD3+ cells) and macrophage (ED-1+ cells) infiltration was performed by blindly counting 10 microscopic fields ($400 \times 400 \mu\text{m}$, 200 $\times$ magnification) in each renal cortex. Infiltrating cell numbers are reported as average of the counts in the 10 fields per renal cortex.

For evaluation of megalin expression, 20 cortical images were taken from each animal at 400 $\times$ magnification. The percentage of cortical area stained positive for megalin was quantified in each image using MetaMorph software (Molecular Devices LLC., San Jose, CA) and the average megalin expression per animal was calculated. The data is reported as the average % area stained positive for megalin per experimental group.

Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assay

Following manufacturer's directions, the Apoptag® Plus Peroxidase *In Situ* Apoptosis Kit (MP Biomedicals, Santa Ana, CA) was used to detect dead cells in renal tissue slides. TUNEL-positive cells in renal cortex were counted in 10 microscopic fields ($400 \times 400 \mu\text{m}$, 200 $\times$ magnification). TUNEL-positive cell counts are reported as average of the counts in the 10 fields per kidney cortical region.

Renal injury marker determination

Albumin and the proximal tubular injury marker NGAL were determined in urine by ELISA (GenWay Biotech, Inc, San Diego, CA, and Abcam, respectively) and urinary excretion

calculated. Renal cortical expression and excretion of KIM-1 were also determined by ELISA (R&D Systems, Minneapolis, MN). Protein excretion by the experimental animals was determined by a Bradford assay (Bio-Rad). Excretion rates were calculated using gravimetrically determined values for 24-hr urine volume collected in metabolic cages at the beginning of the study (normal salt) and at the end of the 3 week period of on high salt diet.

Assessment of ET-1, TNF- α , H₂O₂ and TBARS excretion

ET-1 levels in undiluted urine samples were determined by a chemiluminescent assay (Human ET-1 QuantiGlo kit; R&D Systems). Urinary TNF- α concentration was determined by ELISA (R&D Systems), and H₂O₂ concentration was determined using the Amplex® Red Hydrogen Peroxide/Peroxidase Assay Kit (Thermo Fisher Scientific; Waltham, MA). Urinary concentration of Thiobarbituric Acid Reactive Substances (TBARS) was also assessed by ELISA (Cayman Chemical; Ann Harbor, MI). Excretion rates were determined from 24-hr urine volume collected in metabolic cages at the end of the 3 week period of on high salt diet.

Measurement of glomerular albumin permeability (P_{alb})

Glomeruli were isolated by consecutively passing the isolated renal cortex through a series of filters of decreasing pore size (180, 100 and 75 μ m), as previously described;^{89, 90} the isolation was performed on ice. This technique results in intact and decapsulated glomeruli suspended in ice-cold Hanks balanced salt solution (pH 7.4, Corning, Corning, NY). The freshly isolated glomeruli were affixed to poly-L-lysine-covered wells in a 6 well-plate. Changes in glomerular volume were recorded in response to an oncotic gradient created by changing the concentrations of albumin in the medium. The consequent changes in glomerular volume were measured using CellSens software (Olympus America). Glomerular permeability to albumin, expressed as convectional P_{alb} , was calculated as $P_{alb} = 1 - \text{albumin reflection coefficient}$, as previously described.⁹¹

Isolation of cortical immune cells and flow cytometry

Immune cells were isolated from renal cortical tissue as previously described with minor modifications.⁹² In brief, rats were anesthetized with isoflurane and a catheter was implanted in the abdominal aorta to perfuse the kidneys with saline. The kidneys were then collected and the renal cortex was isolated from the left kidney, minced and digested with collagenase in DMEM for 30 min and 37°C in a shaking water bath. The digestate was then passed 3 times through a 40 μ m filter (ThermoFisher, Waltham, MA) and washed with PEB buffer. Red blood cells were lysis with ACK lysis buffer for 3 min at room temperature and then washed with PBS.

The isolated cortical immune cells were then washed with staining buffer and incubated on ice for 30 minutes with the following mouse-anti rat antibodies: phycoerythrin-conjugated anti-CD45 (CD45-PE, clone OX-1), fluorescein isothiocyanate-conjugated anti-CD3 (CD3-FITC, clone G4.18), allophycocyanin-conjugated anti-CD4 (CD4-APC, clone OX-35), peridinin chlorophyll protein complex-conjugated anti-CD8 (CD8- PerCP, clone OX-8), PE-cyanine 7 anti-CD11b/c (CD11b/c- PE-Cy7, clone OX-42), and peridinin chlorophyll protein complex-conjugated anti-MHC-II (MHC-II- PerCP, clone OX-17). To block the

unspecific binding to the Fc γ 3 receptors, cells were also incubated with mouse anti-rat CD16/32. E450-conjugated fixable viability stain was also added to the samples. All antibodies were purchased from BD Biosciences (San Jose, CA), except MHC-II- PerCP that was purchased from eBiosciences (San Diego, CA). Data acquisition was performed using a LSRII flow cytometer (BD Biosciences) and FACSDiva software (BD Biosciences). Results were analyzed with FlowJo software (Tree Star Inc, Ashland, OR).

Statistical analysis

All data are expressed as mean $\pm$ SEM. Differences between genotypes and treatments were analyzed by two-way analysis of variance with a Tukey's *post hoc* test. MAP was analyzed by two-way repeated measures ANOVA. A *P* value of less than 0.05 was considered statistically significant. All statistical analyses were conducted using GraphPad Prism 6 (GraphPad Software; La Jolla, CA).

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Figure 1.

(a) Urinary excretion of protein and albumin in TG control and ET_B-deficient rats fed normal salt (0.8% NaCl) or high salt (8% NaCl) diet and treated with vehicle or TUDCA. $n = 5-6/\text{group}$. (b) Renal cortical KIM-1 protein expression and urinary excretion of KIM-1, NGAL and nephrin in TG control and ET_B-deficient rats fed normal salt (0.8% NaCl) or high salt (8% NaCl) diet and treated with vehicle or TUDCA. $n = 5-6/\text{group}$. * $P < 0.05$.

Figure 2.

(a) Representative images and quantification of megalin in TG control and ET_B-deficient rats fed high salt (8% NaCl) diet and treated with vehicle or TUDCA. $n = 5-8/\text{group}$. Scale bar = 50 μm . * $P < 0.05$. (b) Average glomerular albumin permeability (P_{alb}) in high salt-fed Wistar Kyoto rats treated with ET_B antagonist in the drinking water and daily injections of vehicle or TUDCA for 3 weeks. * $P < 0.05$. $n = 5/\text{group}$.

Figure 3.

Representative images and quantification of (a) glomerular injury, cortical interstitial fibrosis, and (b) thickness of the glomerular capsule in TG control and ET_B-deficient rats fed high salt (8% NaCl) diet and treated with vehicle or TUDCA. $n = 5-8/\text{group}$. Scale bar = 50 μm . * $P < 0.05$.

Figure 4. Representative images and quantification of brush border integrity in TG control and ET_B -deficient rats fed high salt (8% NaCl) diet and treated with vehicle or TUDCA. $n = 5-8$ /group. Black bar = 50 μ m; white bar = 2mm. * $P < 0.05$.

Figure 5. Representative images of iron deposition (blue dots) in proximal tubules of TG control and ET_B-deficient rats fed high salt (8% NaCl) diet and treated with vehicle or TUDCA. $n = 5-8$ /group. Scale bar = 2mm.

Figure 6.

Representative images and quantification of cell death (TUNEL-positive cells) present in renal cortex of TG control and ET_B-deficient rats fed normal salt (0.8% NaCl) or high salt (8% NaCl) diet and treated with vehicle or TUDCA for 3 weeks. $n = 6/\text{group}$. Bar = 50 μm. * $P < 0.05$.

Figure 7.

Representative images and quantification of cell death (TUNEL-positive cells) present in renal outer medulla of TG control and ET_B -deficient rats fed normal salt (0.8% NaCl) or high salt (8% NaCl) diet and treated with vehicle or TUDCA for 3 weeks. $n = 6/\text{group}$. Bar = 50 μm . * $P < 0.05$.

Figure 8. Representative images of (a) T-lymphocyte (CD3⁺ cells) and (b) macrophage (ED-1⁺ cells) infiltration in renal cortex of TG control and ET_B-deficient rats after 3 weeks of high salt diet and daily treatment with vehicle or TUDCA. *n* = 6/group. Bar = 50 μ m.

Figure 9.

(a) Percentage of total events of leukocytes (CD45⁺), T lymphocytes (CD3⁺), T helper cells (CD3⁺CD4⁺) and T cytotoxic cells (CD3⁺CD8⁺) in renal cortical tissue of high salt-fed Wistar Kyoto rats treated with ET_B antagonist in the drinking water and daily injections of vehicle or TUDCA for 3 weeks. * $P < 0.05$. $n = 5$ /group. (b) Representative flow cytometry dot plots depicting the proportion of CD45⁺, CD3⁺, CD3⁺CD4⁺ and CD3⁺CD8⁺ cells in renal cortical tissue of high salt-fed Wistar Kyoto rats treated with ET_B antagonist in the drinking water and daily injections of vehicle or TUDCA for 3 weeks.

Figure 10.

Excretion of TNF- α (a), hydrogen peroxide (b) and TBARS (c) in TG control and ET_B-deficient rats after 3 weeks of high salt diet and daily treatment with vehicle or TUDCA. $n = 5-6$ /group.

- TG control + 8% NaCl + vehicle ▽ ET_B deficient + 8% NaCl + vehicle
 ● TG control + 8% NaCl + TUDCA ▴ ET_B deficient + 8% NaCl + TUDCA

Figure 11.

Mean arterial pressure (24-hr averages) in TG control and ET_B -deficient rats, monitored by telemetry. Rats were fed normal salt (0.8% NaCl) diet during the first 3 days of recordings and then switched to high salt (8% NaCl) diet for a period of 3 weeks. During the high salt period, rats received daily i.p. injections of vehicle or TUDCA. $n = 4/\text{group}$. * $P < 0.05$ vs. ET_B -deficient treated with vehicle. At all time points, ET_B -deficient rats had higher mean arterial pressure than TG control rats.

Table 1.

Average glomerular surface area and number of glomeruli in TG control and ET_B-deficient rats after 3 weeks of high salt diet and daily treatment with vehicle or TUDCA. $n = 6/\text{group}$.

GLOMERULAR SURFACE AREA (μm^2)			
TG control + 8% NaCl		ET _B -deficient + 8% NaCl	
Vehicle	TUDCA	Vehicle	TUDCA
7761.7 $\pm$ 271.6	7167.2 $\pm$ 204.2	7782.2 $\pm$ 294.1	7254.1 $\pm$ 182.4
NUMBER OF GLOMERULI (in 20 microscopy fields)			
TG control + 8% NaCl		ET _B -deficient + 8% NaCl	
Vehicle	TUDCA	Vehicle	TUDCA
83.0 $\pm$ 2.3	88.3 $\pm$ 2.9	98.0 $\pm$ 8.2	101.3 $\pm$ 5.8

Table 2.

Average numbers of T cells (CD3⁺ cells) and macrophages (ED-1⁺ cells) in renal cortex and outer and inner medulla of TG control and ET_B-deficient rats after 3 weeks of high salt diet and daily treatment with vehicle or TUDCA. *n* = 6/group. **P* < 0.05 vs. same genotype + vehicle; †*P* < 0.05 vs. TG control + vehicle

CORTEX				
	TG control + 8% NaCl		ET_B-deficient + 8% NaCl	
	Vehicle	TUDCA	Vehicle	TUDCA
CD3⁺ cells (cells/field)	10.8 ± 0.5	9.1 ± 1.2	17.4 ± 4.5 [†]	10.8 ± 0.5*
ED-1⁺ cells (cells/field)	6.9 ± 0.7	7.1 ± 1.2	10.5 ± 2.2	6.8 ± 0.6
OUTER MEDULLA				
	TG control + 8% NaCl		ET_B-deficient + 8% NaCl	
	Vehicle	TUDCA	Vehicle	TUDCA
CD3⁺ cells (cells/field)	13.9 ± 1.1	9.1 ± 1.1*	14.0 ± 0.6	9.6 ± 0.8*
ED-1⁺ cells (cells/field)	4.1 ± 0.6	2.8 ± 0.7	4.0 ± 0.9	3.6 ± 0.5
INNER MEDULLA				
	TG control + 8% NaCl		ET_B-deficient + 8% NaCl	
	Vehicle	TUDCA	Vehicle	TUDCA
CD3⁺ cells (cells/field)	9.8 ± 4.8	10.9 ± 3.7	10.5 ± 3.2	5.6 ± 1.6
ED-1⁺ cells (cells/field)	2.4 ± 0.5	1.7 ± 0.5	2.4 ± 0.7	2.3 ± 0.5

Table 3.

Relative mRNA expression of pre-pro-ET-1, ET_A and ET_B receptors, ER stress markers and toll-like receptors 2 and 4 in cortex of TG control and ET_B-deficient rats after 3 weeks of high salt diet and daily treatment with vehicle or TUDCA. *n* = 6–9/group.

	CORTICAL mRNA EXPRESSION			
	TG control + 8% NaCl		ET _B -deficient + 8% NaCl	
	Vehicle	TUDCA	Vehicle	TUDCA
<i>Edn1</i>	1.0 ± 0.3	1.1 ± 0.3	0.5 ± 0.2	0.7 ± 0.3
<i>Ednra</i>	1.0 ± 0.5	2.0 ± 0.7	0.7 ± 0.4	0.9 ± 0.5
<i>Ednrb</i>	1.0 ± 0.4	1.3 ± 0.4	1.0 ± 0.5	0.9 ± 0.4
<i>GRP78</i>	1.0 ± 0.4	1.26 ± 0.2	1.8 ± 0.8	1.3 ± 0.4
<i>ATF-6</i>	1.0 ± 0.4	1.5 ± 0.5	1.1 ± 0.5	1.3 ± 0.5
<i>ATF-4</i>	1.0 ± 0.4	1.0 ± 0.4	1.1 ± 0.5	0.8 ± 0.3
<i>sXBP-1</i>	1.0 ± 0.4	1.2 ± 0.3	1.2 ± 0.5	1.5 ± 0.7
<i>CHOP</i>	1.0 ± 0.3	1.4 ± 0.3	1.4 ± 0.4	1.9 ± 0.5
<i>Caspase-12</i>	1.0 ± 0.4	0.9 ± 0.3	1.9 ± 1.1	1.2 ± 0.6
<i>Tlr2</i>	1.0 ± 0.2	0.9 ± 0.2	1.7 ± 0.3	1.0 ± 0.2
<i>Tlr4</i>	1.0 ± 0.2	1.2 ± 0.4	3.4 ± 1.2	1.9 ± 0.4

Table 4.

Sequences for ER stress and TLR primers used for RT-PCR.

Gene name	Sequence
<i>GRP78</i>	F: AAC CCA GAT GAG GCT GTA GCA ⁹³ R: ACA TCA AGC AGA ACC AGG TCA C
<i>ATF-4</i>	F: TAT GGA TGG GTT GGT CAG TG ⁹⁴ R: CTC ATC TGG CAT GGT TTC C
<i>ATF-6</i>	F: GAT TTG ATG CCT TGG GAG TC ⁹⁴ R: GGA CCG AGG AGA AGA GAC AG
<i>sXBP-1</i>	F: CTG AGT CCG AAT CAG GTG CAG ⁹⁵ R: GGT CTT GTA GAA GGG TAC CTA
<i>CHOP</i>	F: CCA GCA GAG GTC ACA AGC AC ⁹³ R: CGC ACT GAC CAC TCT GTT TC
<i>Caspase 12</i>	F: CAC TGC TGA TAC AGA TGA GG ⁹⁴ R: CCT TCC ATC CGT TCT CAC C
<i>TLR4</i>	F: CCA GAG CCG TTG GTG TAT CT ⁹⁶ R: TCA AGG CTT TTC CAT CCA AC
<i>TLR2</i>	F: GGA GAC TCT GGA AGC AGG TG ⁹⁶ R: CGC CTA AGA GCA GGA TCA AC