

Ouabain Protects against Shiga Toxin–Triggered Apoptosis by Reversing the Imbalance between Bax and Bcl-xL

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ABSTRACT

Hemolytic uremic syndrome, a life-threatening disease often accompanied by acute renal failure, usually occurs after gastrointestinal infection with Shiga toxin 2 (Stx2)–producing *Escherichia coli*. Stx2 binds to the glycosphingolipid globotriaosylceramide receptor, expressed by renal epithelial cells, and triggers apoptosis by activating the apoptotic factor Bax. Signaling via the ouabain/Na,K-ATPase/IP3R/NF- κ B pathway increases expression of Bcl-xL, an inhibitor of Bax, suggesting that ouabain might protect renal cells from Stx2-triggered apoptosis. Here, exposing rat proximal tubular cells to Stx2 *in vitro* resulted in massive apoptosis, upregulation of the apoptotic factor Bax, increased cleaved caspase-3, and downregulation of the survival factor Bcl-xL; co-incubation with ouabain prevented all of these effects. Ouabain activated the NF- κ B antiapoptotic subunit p65, and the inhibition of p65 DNA binding abolished the antiapoptotic effect of ouabain in Stx2-exposed tubular cells. Furthermore, *in vivo*, administration of ouabain reversed the imbalance between Bax and Bcl-xL in Stx2-treated mice. Taken together, these results suggest that ouabain can protect the kidney from the apoptotic effects of Stx2.

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Hemolytic uremic syndrome (HUS) is a life-threatening disease, with acute renal failure as one of the most prominent symptoms. HUS generally occurs after a gastrointestinal infection with Shiga toxin 2 (Stx2)–producing *Escherichia coli*.^{1,2} Stx2 penetrates the intestine, enters the circulation, and binds with high affinity to glycosphingolipid globotriaosylceramide receptors (Gb3), which are expressed in many tissues but are particularly abundant in renal epithelial cells. The Stx2/Gb3 complex is endocytosed and then translocated via the Golgi apparatus and the endoplasmic reticulum to the cytosol, where the toxic A subunit will exert its effects by acting on ribosomes.³ Apoptosis is a major manifestation of Stx2 toxicity, as shown in biopsy samples from patients with HUS and in kidneys from mice inoculated with Stx2-producing versus Stx-nonproducing *E. coli* O157:H7, those treated with Stx2, and Stx2-exposed cells.^{1,4,5}

The mechanism by which Stx2 activates apoptotic pathways is not fully understood, but several lines of evidence suggest that it involves activation of caspase-8 and the intrinsic, mitochondrial pathway. It is well documented that Stx2 increases caspase-8 expression.^{6,7} Caspase-8 may trigger a signaling cascade that results in caspase-3 cleavage and cell death without involving the mitochondrial pathway (the extrinsic apoptotic pathway), or it may

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act by stimulating the apoptotic oligomerizing factors Bax and Bak, which bind to the mitochondrial membrane and make it permeable. This leads to a series of events that also result in caspase-3 cleavage and cell death (the intrinsic apoptotic pathway).⁷ Bax belongs to the Bcl-2 family, which includes both pro- and antiapoptotic factors. Bcl-xL is an antiapoptotic member of the Bcl-2 family and a potent inhibitor of Bax.^{8,9} Little is known about the effect of Stx2 on Bax and Bcl-xL expression. Two independent studies found that the overexpression of Bcl-xL by transient transfection protected from Stx2 B subunit-mediated apoptosis.^{10,11} HUS was previously more common in children, but the recent large outbreak of food-borne Stx2-producing *E. coli* O104:H4 infection suggested that severe HUS might affect adults as well as children. No available therapy protects patients from acute toxin-mediated cellular injury, including apoptosis.^{12,13} Chronic renal affection or failure occurs in up to 10% of patients who survive the acute manifestations of the disease.¹³

Our group has identified a novel signaling system that protects against apoptosis.^{14,15} The signal is activated by the cardiotonic steroid ouabain. The signaling pathway involves interaction between Na,K-ATPase and the inositol 1,4,5-triphosphate receptor (IP3R), triggering of slow intracellular calcium oscillations, and activation of the NF- κ B p65 subunit

(or RelA).^{16–19} Because NF- κ B p65 is known to increase the expression of Bcl-xL,²⁰ we hypothesized that ouabain-triggered Na,K-ATPase signaling may counteract the apoptotic action of Stx2 by upregulation of Bcl-xL and downregulation of Bax. Here we present a series of studies in support of this hypothesis. We show that ouabain in nM concentrations protects Stx2-exposed rat renal epithelial cells from apoptosis by reversing an imbalance between Bax and Bcl-xL. Studies on mice inoculated with Stx2 provide proof of principle and show that treatment with ouabain protects against apoptosis and reverses the imbalance between Bax and Bcl-xL.

RESULTS

Stx2 Binds to Rat Proximal Tubular Cells

Stx binds to the globotriaosylceramide (Gb3) receptor in the plasma membrane in human as well as rodent cells.^{21–23} To examine whether Gb3 is also expressed in primary rat proximal tubular cells (RPTCs), these cells were fixed with 2% paraformaldehyde, exposed to Stx2, and immunostained. As shown in Figure 1, approximately 50% of cells incubated with Stx2, an Stx2-specific antibody, and secondary antibody exhibited a strong immunosignal, both in the presence and the

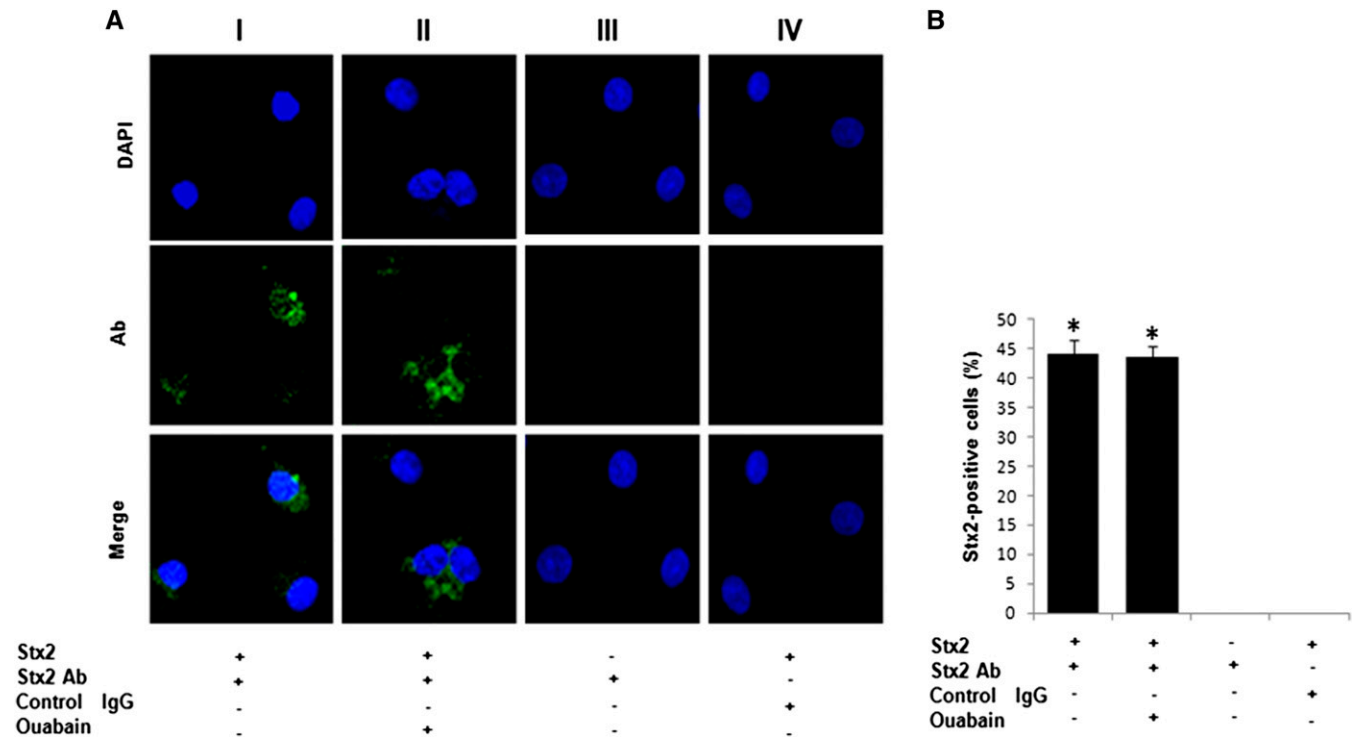


Figure 1. Stx2 binds to rat proximal tubular cells. (A) Panel I: cells exposed to Stx2, Stx2 antibody, and Alexa-conjugated secondary antibody (Ab). Stx2 immunostaining is shown in green. Panel II: cells exposed to Stx2, ouabain, Stx2 antibody, and Alexa-conjugated secondary antibody. Stx2 immunostaining is shown in green. Panel III: cells not exposed to Stx2 but exposed to the anti-Stx2 antibody and secondary antibody. Panel IV: cells not exposed to Stx2, control IgG antibody, and secondary antibody. All cells are counterstained with DAPI (blue). Cells were observed with fluorescent microscope using 40 \times /1.2NP water-immersion objective. For lower magnification, see Supplemental Figure 4. (B) Histograms represent means \pm SEM. * P <0.001. Statistical analysis was performed using the Mann-Whitney U test. Experiments were repeated three times.

absence of ouabain. No signal was detected when the primary antibody was omitted or replaced with a control IgG antibody.

Stx2 2 Induces Apoptosis in Rat Proximal Tubular Cells, and Ouabain Has a Protective Effect

The apoptotic effect of Stx2 was first studied in RPTCs, which 2 days after plating were incubated with standard medium containing Stx2 or Stx2 and ouabain. Cells incubated with standard medium without these additives served as controls. Three days after plating, the cells were prepared for terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining for semiquantitative assessment of the level of apoptosis (Figure 2). In control cells, the mean \pm SEM apoptotic index was $2.1\% \pm 0.2\%$. Stx2, 4 ng/ml, caused extensive apoptosis (apoptotic index, $19.6\% \pm 4.6\%$). In cells co-incubated with Stx2 and ouabain, 5 nM, the apoptotic effect was almost completely abolished (apoptotic index, $2.7\% \pm 0.4\%$). In pilot studies, a Shiga toxin 2 concentration of 4 ng/ml had pronounced and reproducible apoptotic effects (Supplemental Figure 1). This concentration was therefore used in all subsequent experiments.

To further examine the nature of cell death caused by Stx2 exposure, flow cytometry analysis was performed (Figure 3). Cells were treated as described above. Annexin V was used as a marker of early apoptosis, and 7-aminoactinomycin D (7-AAD) as a marker of late apoptosis/necrosis. The number of cells that exhibited an annexin V signal was significantly

($P < 0.05$) higher in cells exposed to Stx2 alone ($19.9\% \pm 0.7\%$) than in control cells ($7.3\% \pm 0.6\%$) and cells exposed to Stx2 and ouabain ($9.7\% \pm 0.9\%$). In a similar fashion, the number of cells that exhibited a 7-AAD signal was also significantly ($P < 0.05$) higher in cells exposed to Stx2 alone ($6.5\% \pm 0.1\%$) than in control cells ($1.0\% \pm 0.4\%$) and cells exposed to Stx2 and ouabain ($1.2\% \pm 0.1\%$).

NF- κ B p65 Subunit Activity

Low concentrations of ouabain activate the survival factor p65,^{20,24} a subunit of the pleiotropic transcriptional factor NF- κ B. Because NF- κ B units under nonstimulated conditions are located in the cytoplasm, we immunostained RPTCs with a p65-specific antibody and compared the nuclear/cytoplasmic ratio of the immunosignal. The ratio was similar in control cells and in cells exposed to Stx2 alone (0.57 ± 0.04 and 0.53 ± 0.03 , respectively) but was significantly higher in cells exposed to Stx2 and ouabain (3.9 ± 0.2) than in the other groups (Figure 4, A and B). We then determined the p65 DNA-binding activity in nuclear extracts from RPTCs; we found that it was significantly reduced in cells exposed to Stx2 compared with control cells but similar in cells co-incubated with Stx2 and ouabain and in control cells (Figure 4C). To test the functional role of NF- κ B activation, the anti-apoptotic effect of ouabain on Stx2-exposed RPTCs was determined in the presence or absence of helenalin, a specific inhibitor of the transcriptional effect of the NF- κ B (Figure

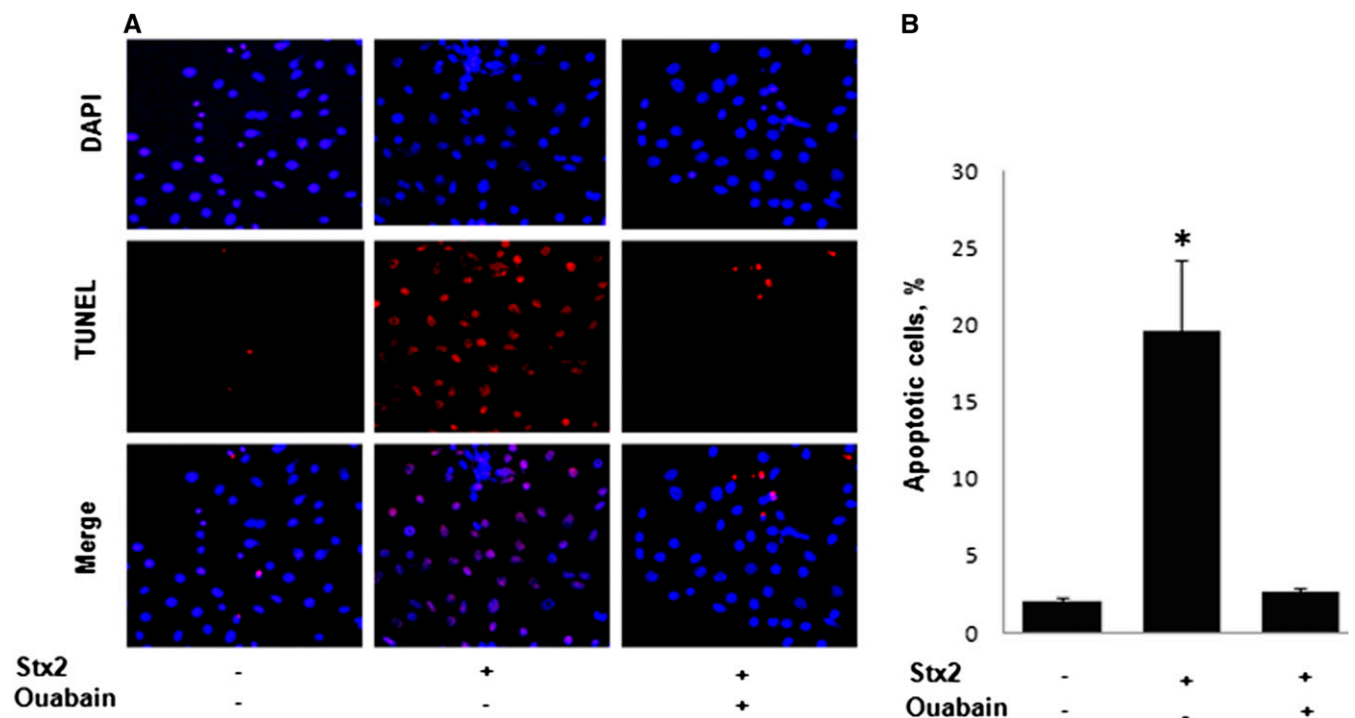


Figure 2. Stx2 induces apoptosis in rat proximal tubular cells, and ouabain has a protective effect. (A) RPTCs were TUNEL-stained (red) to detect apoptotic cells and counterstained with DAPI (blue). Apoptotic index was determined by analyzing five to seven randomly selected areas with 100–200 cells in each area. (B) Histograms represent means \pm SEM. * $P < 0.001$ for Stx2 versus control, Stx2 versus Stx2 plus ouabain. Statistical analysis was performed using the Mann-Whitney U test. Experiments were repeated eight times.

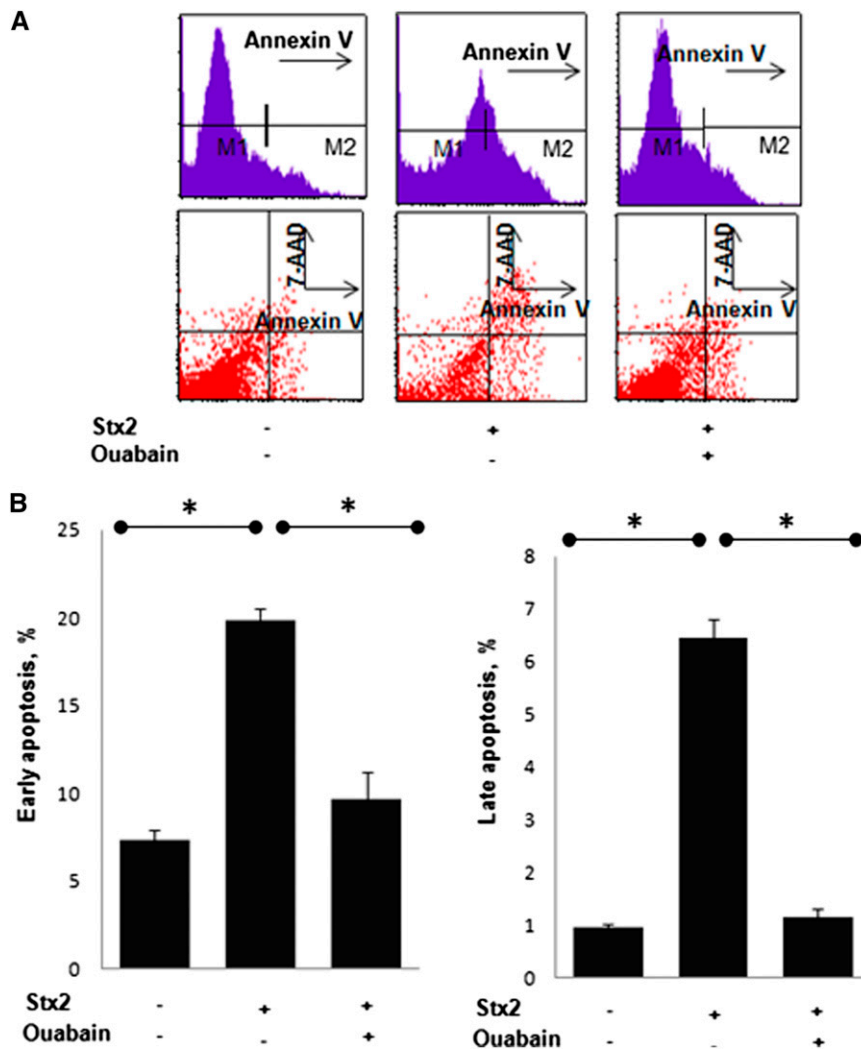


Figure 3. Ouabain increases cell viability. (A) Representative histogram and scatter plots of flow cytometer analysis from different experimental groups. Normal cells (in gate M1) and total apoptotic cells (in gate M2) are shown in the histograms; the early apoptosis (the lower right quadrants) and the late apoptosis and/or dead cells (the upper right quadrant) are shown in the scatter plots. (B) Histograms represent means \pm SEM. * P <0.05. Statistical analysis was performed using the Mann-Whitney U test. Experiments were repeated three times.

4D).^{25,26} Preincubation with helenalin completely abolished the antiapoptotic effect of ouabain in Stx2-exposed cells.

Expression of Bcl-xL, Bax, Caspase-8, and Caspase-3 in Stx2- and Ouabain-Treated Cells

The expression of apoptotic and antiapoptotic factors was determined with immunoblotting. After 16 hours of incubation of RPTCs with Stx2 alone, the expression of Bcl-xL was decreased by $23.7\% \pm 5.9\%$ compared with control cells. In contrast, Bcl-xL expression was similar in cells co-incubated with Stx2 and ouabain and in control cells. In cells exposed to Stx2 alone, expression of Bax was increased by $37.0\% \pm 5.2\%$, but no such increase was observed in cells exposed to Stx2 and ouabain. Bax can initiate a cascade of events that eventually

involves cleavage of caspase-3. The expression of cleaved caspase-3 was significantly increased ($48.1\% \pm 5.4\%$) in cells exposed to Stx2 alone compared with control cells, but no such increase was observed in cells exposed to Stx2 and ouabain. The apoptotic factor caspase-8, an activator of Bax,²⁷ was increased with $42.7\% \pm 5.1\%$ in cells exposed to Stx2 alone and with $38.4\% \pm 5.0\%$ in cells exposed to Stx2 and ouabain (Figure 5, A–D). The effects of Stx2 incubation on Bcl-xL and Bax were significant at 6 hours but were not significant in comparison with control cells for caspase-3 and caspase-8 (Figure 5, E–H). Incubation with ouabain alone caused an increase in Bcl-xL and decrease in Bax expression after 16 hours of incubation (Supplemental Figure 2).

Ouabain Protects Kidneys from Apoptosis in Stx2-Treated Mice

To study the *in vivo* effects of Stx2 and ouabain treatment, mice were given an intraperitoneal injection of Stx2 (285 ng/kg) ($n=9$) or PBS ($n=9$) at day 0. A mini-pump that delivered ouabain (15 $\mu\text{g}/\text{kg}$ per day) or PBS was inserted subcutaneously 1 day earlier. Mice given an intraperitoneal injection of PBS ($n=6$) and receiving PBS *via* mini-pumps ($n=6$) were controls. The mice were observed for 2 days and then euthanized. Mice injected with this dose of Stx2 usually develop symptoms after 3–4 days.^{4,28} Mice were euthanized before symptoms developed in order to detect early apoptotic changes.

Kidneys were immediately removed, fixed, and stained for evaluation of the apoptotic index or for evaluation of Bcl-xL and Bax expression. Two sections from each kidney were used for TUNEL assay of the apoptotic index, which was determined in the renal cortex as the number of TUNEL-positive tubular cells in relation to the total number of tubular cells (Figure 6A). The apoptotic index was significantly higher in mice given Stx2 alone ($n=9$) than in mice given Stx2 and ouabain ($n=9$) ($6.1\% \pm 0.6\%$ and $1.7\% \pm 0.4\%$, respectively, P <0.001). In the control group the apoptotic index was $0.48\% \pm 0.06\%$ (Figure 6A).

The expression of Bcl-xL and Bax was visualized by immunofluorescence. Renal cortical expression of Bcl-xL and Bax was detected in all groups studied (Figure 6B). Semi-quantitative evaluation of the fluorescence by ImageJ software was performed on one section from each kidney. In mice inoculated with Stx2 and receiving PBS from the mini-pump, the Bcl-xL signal was significantly lower ($22.5\% \pm 2.6\%$) than in

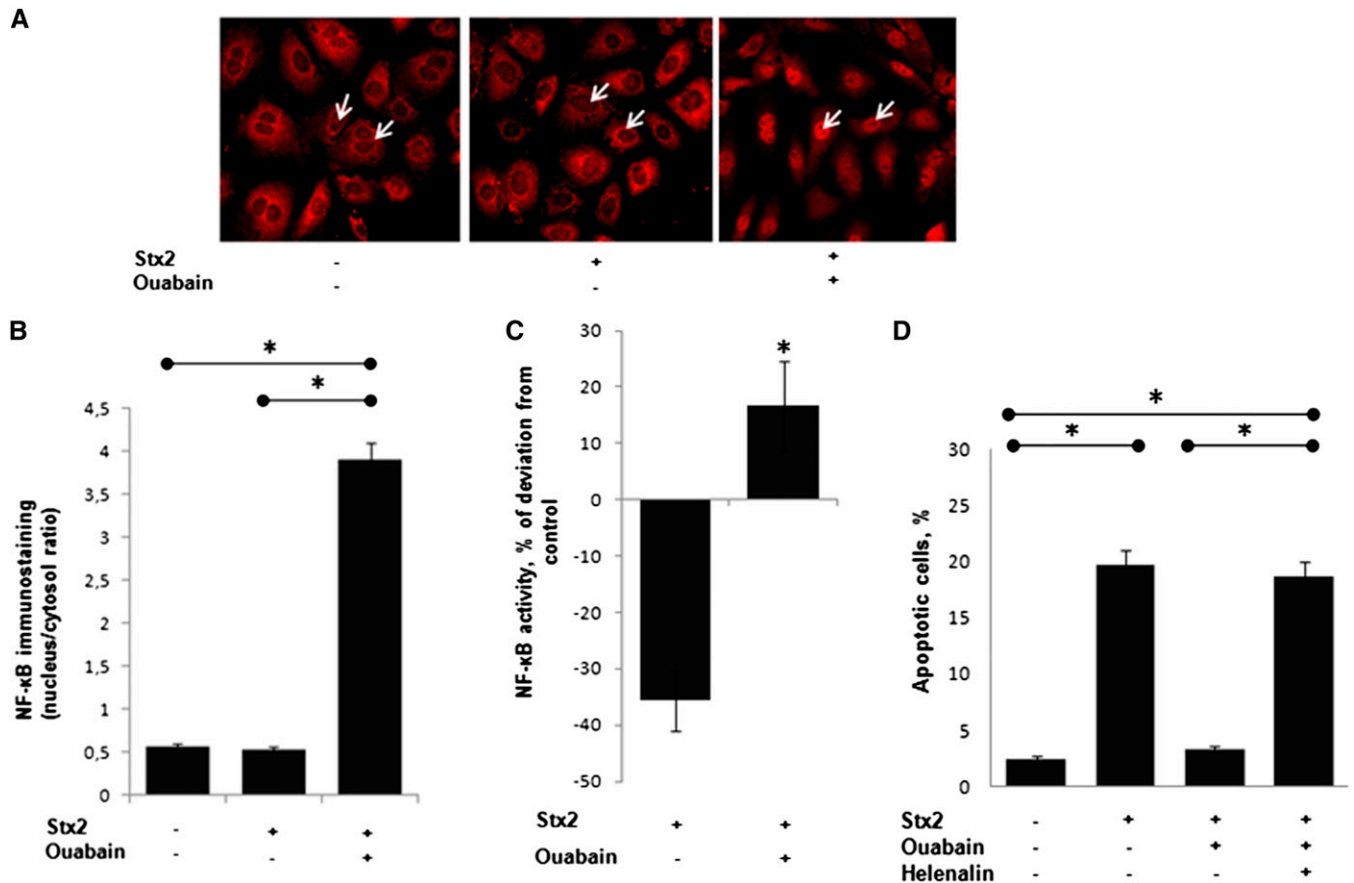


Figure 4. Ouabain activates NF- κ B p65 subunit. (A) Representative confocal images of NF- κ B p65 subunit immunofluorescence signal in RPTCs (white arrows). Note the strong nuclear signal in ouabain-exposed cells in the right panel. (B) Bars show mean nuclear/cytoplasmic NF- κ B p65 subunit signal. Each experiment analyzed 30–50 cells. Histograms represent means \pm SEM. $*P < 0.001$ for Stx2 plus ouabain versus control, Stx2 plus ouabain versus Stx2. Experiments were repeated four times. (C) NF- κ B p65 subunit activity in rat RPTCs determined as NF- κ B DNA-binding capacity in nuclear protein extracts. The activity of the control was set to 100%. Histograms represent means \pm SEM. $*P < 0.01$. Statistical analysis was performed using the one-way ANOVA followed by *post hoc* test. Experiments were repeated three times. (D) Helenalin is a specific NF- κ B inhibitor that abolishes the antiapoptotic effect of ouabain. Apoptotic index was determined by analyzing five to seven randomly selected areas with 50–200 cells, enabling an analysis of around 5000 cells. Histograms represent means \pm SEM. $*P < 0.01$. Experiments were repeated three times.

kidneys from the mice in the control group. In contrast, the intensity of the fluorescent signal from Bcl-xL was similar in Stx2-inoculated mice treated with ouabain and in control mice. Kidneys from control mice exhibited low levels of Bax expression. In mice inoculated with Stx2 alone, expression of Bax was increased by $46.1\% \pm 7.1\%$. Bax was not upregulated in kidneys from mice treated with Stx2 and ouabain. The mice receiving an intraperitoneal injection of PBS and ouabain in the mini-pump did not differ from the control group with regard to any measures studied (Figure 6B).

Ouabain Protects against Podocyte Depletion and Attenuates Renal Function Impairment in Stx2-Treated Mice

The visceral epithelial cells in the glomeruli, the podocytes, are of critical importance for the filtering process but have a limited capacity to regenerate. Podocyte depletion is one of

the major mechanisms behind progressive kidney disease. The number of podocytes was counted in the control group and the two groups inoculated with Stx2. The number of podocytes in 12–15 glomeruli from two kidney sections from each animal included in the study was reduced by 25.2% ($P < 0.01$) in the Stx2 group receiving vehicle compared with the control group but was significantly less reduced (7.7%) in the group receiving ouabain (Figure 7A). Stx2-treated mice with vehicle had a moderate but significant increase in plasma creatinine. This was not observed in Stx2-inoculated mice treated with ouabain (Figure 7B).

DISCUSSION

Shiga toxin is an important virulence factor of *E. coli* strains that are associated with HUS, and Stx2-triggered apoptosis is considered one of the causes of renal damage during HUS.^{1,2}

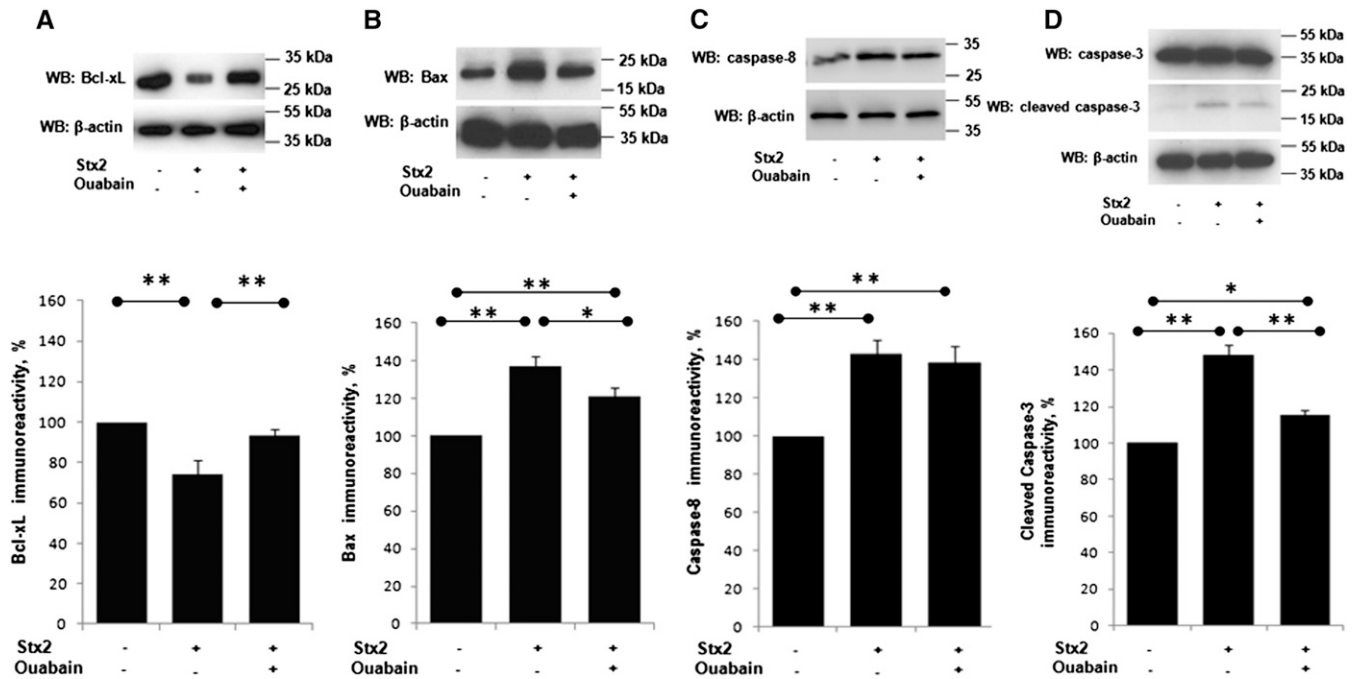


Figure 5. Ouabain regulates the balance between apoptotic and antiapoptotic factors in Stx-2-treated RPTCs. Panel I: Expression of the Bax (A), Bcl-xL (B), caspase-8 (C), and caspase-3 (D) in the presence of Stx2 and ouabain during 16 hours. Densitometric quantification of bands was done for the respective blots. The density of the band from control cells was set to 100%. Histograms represent means \pm SEM. $**P < 0.01$, $*P < 0.05$. Statistical analysis was performed using the Mann-Whitney *U* test. Experiments were repeated five times. Panel II: Expression of the Bax (A), Bcl-xL (B), caspase-8 (C), and caspase-3 (D) in the presence of Shiga toxin 2 and ouabain during 6 hours. Densitometric quantification of bands was done for the respective blots. Histograms represent means \pm SEM. $*P < 0.05$. Statistical analysis was performed using the Mann-Whitney *U* test. Experiments were repeated five times.

The results from this study confirm the apoptotic effect of Stx2 on renal epithelial cells and indicate that the apoptosis is due to an imbalance between the apoptotic factor Bax and the antiapoptotic factor Bcl-xL. The cardiotonic steroid ouabain reverses the imbalance and protects against this manifestation of Stx2 pathology.

Apoptosis is induced *via* two major pathways: the extrinsic pathway, where activation of plasma membrane “death receptors” leads to the recruitment of caspase-8,²⁹ and the intrinsic pathway (the mitochondrial pathway), where the proapoptotic dimerizing factors Bax and Bak are recruited to the mitochondrial membrane.³⁰ Both pathways will initiate signaling cascades that result in the cleavage of caspase-3 and cell death.²⁹ Here we found, in line with several previous studies, that Stx2 exposure for 16 hours caused a large upregulation of caspase-8,⁶ of Bax,^{9,31} and of caspase-3.⁶ We also recorded a substantial downregulation of Bcl-xL. The recordings made after 6 hours of Stx2 exposure indicated that the effects on Bax and Bcl-xL preceded the effects on caspase-8 and -3. Ouabain attenuated the upregulation of Bax, but not of caspase-8. Caspase-8 can initiate both the extrinsic and the intrinsic apoptotic pathway because it may bypass the mitochondrial pathway and directly cleave caspase-3, or, *via* a series of events, facilitate the translocation of Bax to the mitochondrial membrane.²⁹ Taken together, these results imply that in Stx2-triggered apoptosis, caspase-8 may mainly act by stimulating Bax and

the intrinsic apoptotic pathway. Little is known about the effect of Stx2 on the expression of the Bax inhibitor Bcl-xL, but overexpression of Bcl-xL by transient transfection was reported to protect against Stx-triggered apoptosis.¹⁰ Here we could show that the downregulation of this survival factor in Stx2-treated renal epithelial cells was almost completely abolished by ouabain treatment. Because there is little evidence for a more direct interaction between caspase-8 and Bcl-xL, we conclude that the antiapoptotic effect of ouabain is due to inhibition of the intrinsic, mitochondrial apoptotic pathway and that treatment with ouabain protects against apoptosis by reversing an imbalance between Bcl-xL and Bax, as shown in both the *in vitro* and *in vivo* studies.

We ascribe the antiapoptotic effect of ouabain to the signaling function of Na,K-ATPase. Exposure of renal cells to low concentrations of the highly specific Na,K-ATPase ligand, ouabain, will trigger a signaling cascade that involves interaction between the catalytic subunit of Na,K-ATPase and the inositol 1,4,5-triphosphate receptor, intracellular calcium oscillations and activation of the NF- κ B p65 subunit (Supplemental Figure 3). We showed that Stx2 does not affect the capacity of ouabain-bound Na,K-ATPase to interact with IP3R. Bcl-xL is a target for the transcriptional effect of the NF- κ B p65 subunit.³² The calcium oscillations triggered by ouabain/Na,K-ATPase/IP3R signaling have a frequency of approximately 1 peak every 3–5 minutes, and calcium

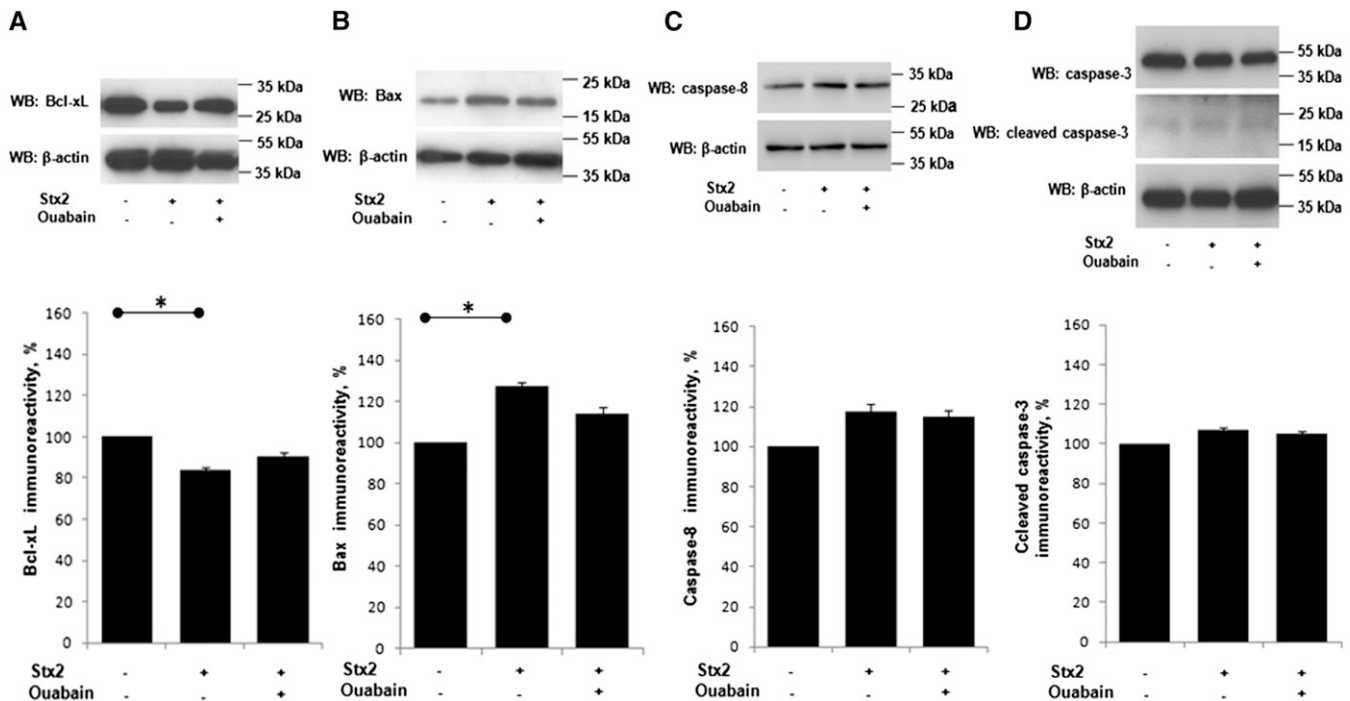


Figure 5. Continued.

oscillations with this periodicity have previously been reported to activate NF- κ B.^{16–19} Here we showed that ouabain signaling triggers activation and translocation of NF- κ B p65 subunit to the nucleus in cells exposed to Stx2 and ouabain but found no evidence of NF- κ B p65 subunit activation in cells exposed to Stx2 alone. Helenalin inhibits the transcriptional effect of NF- κ B p65 subunit; in cells co-incubated with Stx2, ouabain, and helenalin, the capacity of ouabain to protect against apoptosis was lost. The concentration of ouabain used in this study, 5 nM, has no measurable effect on the pumping function of Na,K-ATPase in rat cells.^{18,19}

The *in vivo* study provided proof of principle that ouabain can protect proximal tubular cells from Stx2-triggered apoptosis, that Stx2 triggers an imbalance between Bax and Bcl-xL, and that ouabain treatment can reverse this change in balance. Continuous treatment with ouabain was started 24 hours before the Stx2 injection. This time frame may be relevant for a future therapeutic use of ouabain because patients with Stx2-producing *E. coli* infection seek care after the onset of hemorrhagic diarrhea and before or at the onset of Stx2-triggered pathology. Loss of podocytes is a hallmark of permanent renal damage because these cells have a very low capacity to regenerate.³³ The number of podocytes decreased in both groups of Stx2-inoculated mice, but this reduction was significantly less pronounced in the ouabain-treated group. Taken together, these results imply that the antiapoptotic effect of ouabain will, at least partially, protect against acute and permanent renal damage caused by exposure to Stx2.

The results from this study may have implications that go beyond the treatment of HUS-associated renal apoptosis. Many

disease conditions in the kidney are associated with excessive apoptosis, such as diabetic nephropathy,^{34,35} glomerular tubular disconnection in diseases with profound proteinuria,³⁶ polycystic kidney disease,³⁷ and AKI.³⁸ Expression of Bax is increased in many of these conditions, leading to the suggestion that the Bax inhibitor Bcl-xL would be a potential therapeutic target in renal diseases associated with apoptosis.³⁹ Almost all of the available antiapoptotic drugs have serious adverse effects. The use of caspase inhibitors is for the most part prohibited because of the risk of preventing a physiologic apoptotic process in premalignant cells. However, in contrast to caspase inhibitors, which would completely block the apoptotic pathway, ouabain would, by triggering a signal that results in physiologic upregulation of Bcl-xL, reset and normalize the balance between apoptotic and antiapoptotic factors.

CONCISE METHODS

Cells

RPTCs were prepared from kidneys of 20-day-old male Sprague-Dawley rats as described previously. Cells were plated on 12-mm glass coverslips in 24-well cell culture plates. On day 2 *in vitro*, at which time they have been shown to maintain most of their phenotype as RPTCs, the cells were exposed to the indicated compounds (Stx2 with or without ouabain) or to vehicle (PBS).

Stx2 Binding to RPTCs

RPTCs were plated on 12-mm glass coverslips in 24-well cell culture plates. The cells were fixed in paraformaldehyde, washed once with cold

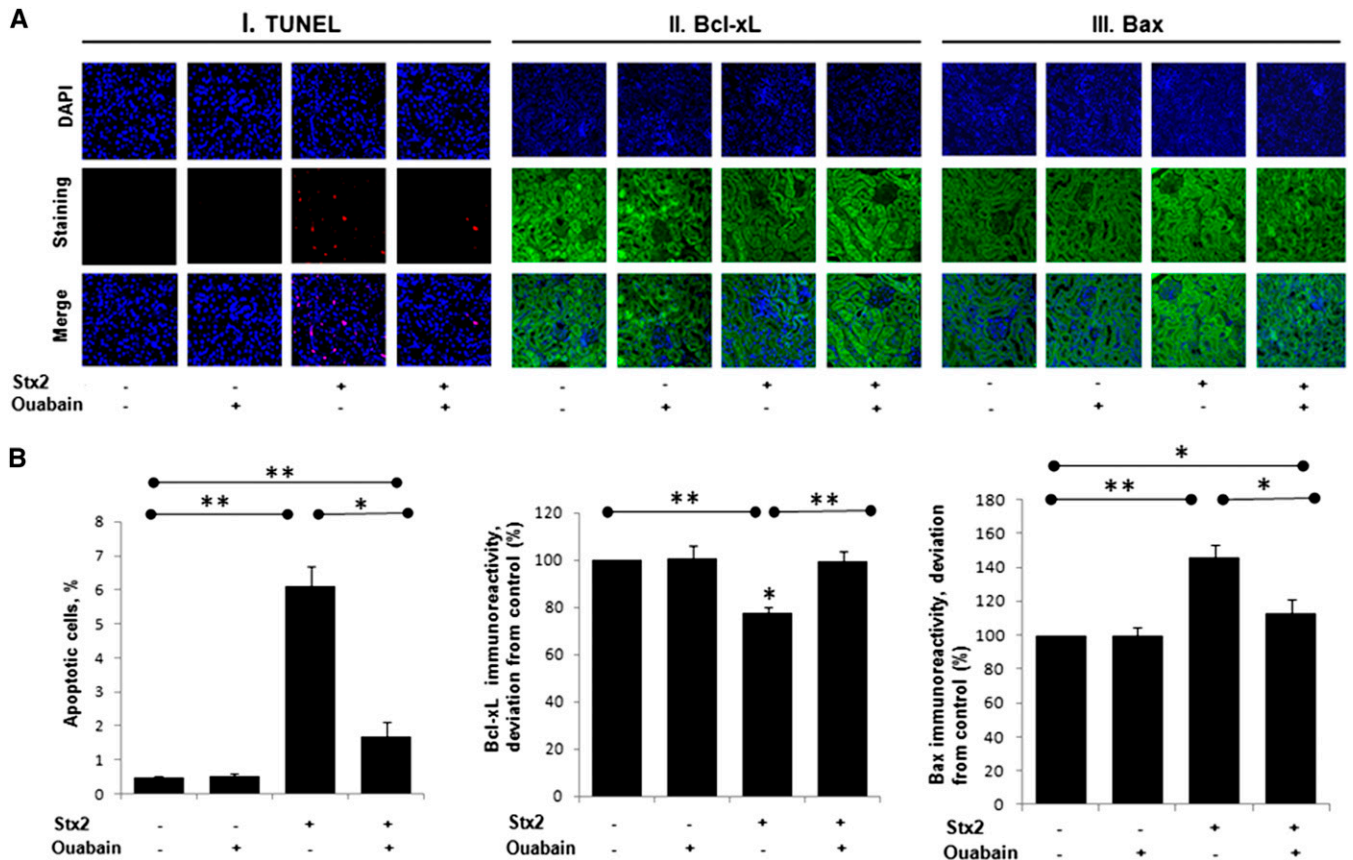


Figure 6. Ouabain protects kidneys from apoptosis in Stx2-treated mice. (A) Representative confocal images of kidney sections from mice inoculated with Stx2 or PBS as vehicle, with and without continuous treatment with ouabain. Sections were labeled with TUNEL (red) to detect apoptotic cells (I), immunohistochemically stained for Bcl-xL (II), and Bax (III). Nuclei were counterstained with DAPI (blue). (B) Apoptotic index is given as percentage apoptotic cells and was determined by analyzing 5–7 randomly selected areas, with 80–150 cells in each area, from two independent experiments. Immunoreactivity for Bcl-xL and Bax is expressed as percentage deviation from control. Histograms represent means \pm SEM. Statistical analysis was performed using the Mann-Whitney *U* test. * $P < 0.05$, ** $P < 0.001$.

PBS, and incubated with Stx2 for 1 hour with gentle shaking. Cells were then incubated with mouse monoclonal anti-Stx2 IgG1 antibody or the isotype control antibody, followed by incubation with Alexa Fluor 488 fluorescence-conjugated goat antimouse IgG antibody.

Detection of Apoptotic Cells

RPTCs were plated on 12-mm glass coverslips in 24-well cell culture plates. On culture day 2, when cells had achieved approximately 50% confluence, indicated compounds were added to the medium. The ApopTag Red In Situ Apoptosis Detection kit was used to determine apoptotic cells. TUNEL assay was conducted according to the manufacturer’s instructions. Nuclei were counterstained with DAPI. Cells were mounted in Immu-Mount and images were recorded with confocal microscopy. The apoptotic index was calculated as the percentage of TUNEL-positive cells; the total number of cells was determined by DAPI stain.

FACS Staining

Approximately 1 million RPTCs from each study group were harvested, washed, and labeled with fluorochrome-conjugated annexin V (an indicator of early apoptosis) and 7-AAD (viability

dye) in the dark at room temperature for 15 minutes using Annexin V-PE Apoptosis detection Kit I. This procedure was followed by FACSCalibur analysis. The quantification analysis was performed with CellQuest software, version 3.3.

NF- κ B p65 Subunit Activity

NF- κ B p65 subunit translocation to nucleus was used as an index of NF- κ B p65 subunit activation. RPTCs were labeled with rabbit polyclonal NF- κ B p65 antibody, and secondary antibody was antirabbit Alexa 546. Images of immune-labeled cells were recorded with confocal microscopy. The ratio of the immunosignal between nucleus and cytosol was measured using ImageJ software. An area corresponding to 90% of the nucleus and an identical area in the cytoplasm were used for the measurements. NF- κ B p65 subunit DNA binding in nuclear protein extracts was assessed using a commercially available NF- κ B p65 transcription factor assay according to the manufacturer’s instructions.

The ApopTag Red In Situ Apoptosis Detection kit was used to determine apoptotic cells. TUNEL assay was conducted according to the manufacturer’s instructions. RPTCs were preincubated with helenalin, an NF- κ B inhibitor (1 μ M). Cells were mounted in Immu-Mount, and images were recorded with confocal microscopy. The

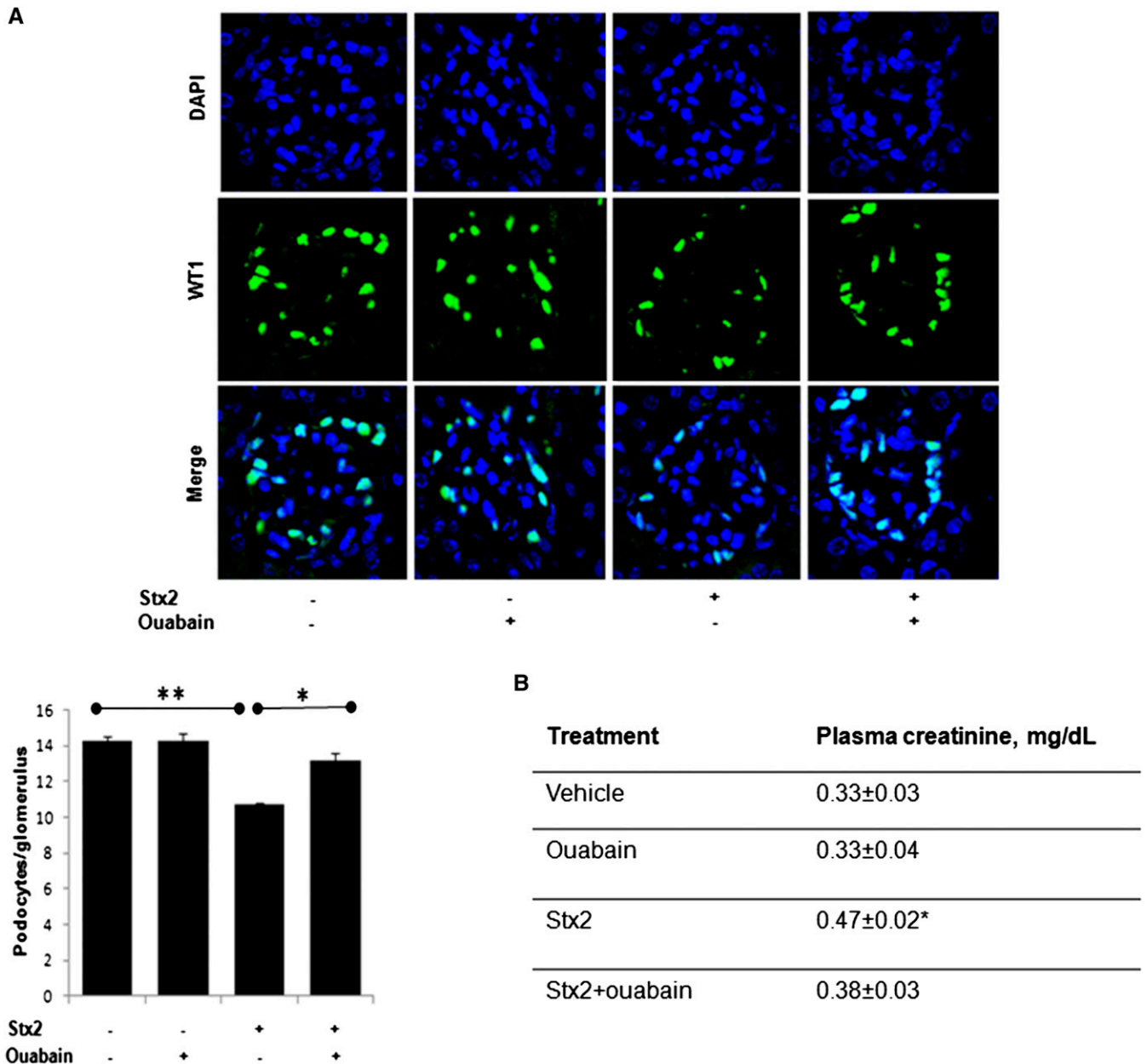


Figure 7. Ouabain prevents podocyte loss and attenuates renal function impairment. (A) Representative micrographs demonstrate podocytes from mice treated with Stx2 or PBS as vehicle, with and without continuous treatment with ouabain. WT-1 staining has been used to recognize podocytes. Histograms represent means \pm SEM. ** $P < 0.01$, * $P < 0.05$. Statistical analysis was performed using the Mann-Whitney U test. (B) Plasma creatinine values in Stx2- and ouabain-treated mice. Data presented as means \pm SEM. * $P < 0.05$ for Stx2 versus control, ouabain, and Stx2 plus ouabain. Statistical analysis was performed using the Mann-Whitney U test.

apoptotic index was calculated as the percentage of ApopTag-positive cells; the total number of cells was determined by DAPI stain.

Immunoprecipitation of NKA and IP3R

After incubation with Stx2 and ouabain for 24 hours, RPTCs were washed twice with PBS, lysed on ice in cold buffer, sonicated, and centrifuged. Immune complexes were then incubated with 30 $\mu\text{g/L}$ of 50% slurry of protein A/GPLUS-agarose beads. On the next day the beads were processed for SDS-PAGE and immunoblotting.

The protein bands were visualized by chemiluminescence using secondary antibodies labeled with horseradish peroxidase. Densitometric quantification of films was done, correction for total protein was made, and control values were set to 100%.

Immunoblotting for Detection of Bax, Bcl-xL, Caspase-3, and Caspase-8

Proteins solubilized in Laemmli sample buffer were resolved in polyacrylamide gels by SDS-PAGE and transferred to a polyvinylidene

difluoride membrane. The protein bands were visualized by chemiluminescent substrate.

Animal Studies

Experiments were conducted on C57BL/6 male mice weighing 22–24 g. Mice received an intraperitoneal injection of Shiga toxin 2 at 285 ng/kg. PBS injection was used for control mice. Ouabain was delivered in a dose corresponding to 15 μ g/kg per day using subcutaneous neck mini-pumps. Controls received PBS vehicle in the pump. All mice were euthanized at day 3 after inoculation with Stx2. Mouse kidneys were removed and fixed in 4% paraformaldehyde in PBS (pH, 7.4) immediately after euthanasia. Tissues were then embedded in paraffin and sectioned.

TUNEL Assay

Renal tissue (3- μ m sections) used for TUNEL assay was deparaffinized and rehydrated before processing. Two sections from each mouse kidney were used for TUNEL assay. The ApopTag Red In Situ Apoptosis Detection kit was used to determine the apoptotic index. TUNEL assay was conducted according to the manufacturer's instructions. Nuclei were counterstained with DAPI. Sections were mounted in Immu-Mount and recorded with confocal microscopy. Cells were considered apoptotic when they exhibited ApopTag Red staining and characteristic apoptotic structure.

Apoptotic index was calculated as the percentage of TUNEL-positive cells; the total number of cells was determined by DAPI stain. In each slice, five to seven randomly selected areas were examined, and in each area, between 80 and 150 DAPI-stained cells were counted.

Immunohistochemistry

Renal tissue (3- μ m sections) was deparaffinized and rehydrated before processing. Antigens were retrieved by boiling in citrate buffer. Sections were treated with Triton X-100 0.3% in PBS for 20 minutes. After three PBS washes, sections were incubated with blocking buffer (5% bovine serum albumin and Triton X-100 0.1% in PBS) for 1 hour. The rabbit polyclonal anti-Bcl-xL primary antibody and rabbit polyclonal anti-Bax were applied overnight at 4°C. After three PBS washes, sections were incubated with a secondary Alexa Fluor 488 goat antirabbit IgG for 1 hour at room temperature. Nuclei were counterstained with DAPI. All samples were stained using identical protocols. Sections were mounted in Immu-Mount and images were recorded with confocal microscopy. All microscopy recordings were done during one session using identical microscope parameters. Image analysis was performed using ImageJ software. Three areas in each section were analyzed.

For podocyte immunostaining, the rabbit polyclonal anti-Wilms tumor-1 (WT1) primary antibody was applied overnight to kidney sections at 4°C. Controls were performed in the same conditions, but the primary antibody was omitted. After three PBS washes, sections were incubated with a secondary antirabbit Alexa Fluor 488 IgG. Nuclei were counterstained with DAPI. The immune-labeled cells were observed with Zeiss LSM 510 laser scanning confocal microscope using a 63 \times /1.2NA oil-immersion objective. For quantitative determination of podocyte numbers, the WT-1-positive cells were

counted in at least 12–15 randomly chosen glomeruli. Two sections from each kidney were analyzed.

Analyticals

Blood was collected by cardiac puncture of anesthetized animals. Plasma creatinine was determined using a Mouse Creatinine (Cr) ELISA kit (Cusabio, Biotech Co., LTD, China). Reaction was analyzed on automated spectrophotometer (Wallac Victor³ 1420 Multilabel Counter).

Statistical Analyses

Statistical analysis was performed with Statistica 6.0 software (Statsoft, Tulsa, OK) and SAS statistical software, version 9.1 (SAS Institute, Cary, NC). A *t* test was used to compare quantitative variables between groups if the distribution was parametric; ANOVA followed by the *post hoc* test and nonparametric test (Mann-Whitney *U* test) were used to test significance of differences. Statistical significance was determined as *P*<0.05. Values are expressed as means \pm SEM.

A complete description of the Methods is provided in the Supplemental Material.

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