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ORIGINAL ARTICLE

Gabriela Verónica Camerano · Oscar David Bustuoabad · Roberto Pablo Meiss · Sonia Alejandra Gómez · Gabriela Cristina Fernández · Martín Amadeo Isturiz · Marina Sandra Palermo · Graciela Isabel Dran

Compensatory renal growth protects mice against Shiga toxin 2-induced toxicity

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Abstract Uninephrectomy (Unx) is followed by the compensatory renal growth (CRG) of the remaining kidney. Previous evidence has shown that during CRG, renal tissue is resistant to a variety of pathologies. We tested the hypothesis that the functional changes that take place during CRG could attenuate Shiga toxin (Stx) toxicity in a mouse model of Stx2-induced hemolytic uremic syndrome (HUS). The participation of nitric oxide (NO) was analyzed. After CRG induction with Unx, mice were exposed to a lethal dose of Stx2, and the degree of renal damage and mortality was measured. Stx2 effects on the growth, renal blood flow (RBF) and NO synthase (NOS) intrarenal expression in the remaining kidney were then studied. The induction of CRG strongly prevented Stx2-mediated mortality and renal damage. Administration of the NOS inhibitor N^G-nitro-L-arginine methyl ester (L-NAME) during CRG partially impaired the protection. Both Stx2 and L-NAME interfered with the hypertrophic and hyperplastic responses to Unx, as well as with the increase in RBF. In intact mice, Stx2 decreased renal perfusion, inhibited endothelial NOS basal expression and enhanced inducible NOS expression; all of these effects were attenuated by prior Unx. It is concluded that during CRG mice are highly protected against Stx2 toxicity and lethality. The protective capacity of CRG could be related to the enhancement of renal perfusion and preservation of eNOS renal expression, counterbalancing two major pathogenic mechanisms of Stx2.

Keywords Uninephrectomy · Compensatory renal growth · Shiga toxin · Hemolytic uremic syndrome

G. V. Camerano · O. D. Bustuoabad · G. I. Dran
Sección Medicina Experimental,
Academia Nacional de Medicina,
Buenos Aires, Argentina

R. P. Meiss
Departamento de Patología, Centro de Estudios Oncológicos,
Academia Nacional de Medicina,
Buenos Aires, Argentina

S. A. Gómez · G. C. Fernández · M. A. Isturiz · M. S. Palermo
Sección Inmunología, Academia Nacional de Medicina,
Buenos Aires, Argentina

G. V. Camerano (✉)
División Medicina Experimental, ILEX,
Academia Nacional de Medicina,
Pacheco de Melo 3081,
C1425 AUM Buenos Aires, Argentina
e-mail: gdran@hematologia.anm.edu.ar
Tel.: +54-11-48053411
Fax: +54-11-48039475

Introduction

Unilateral nephrectomy (Unx) results in the compensatory growth of the remaining kidney. This compensatory renal growth (CRG) consists predominantly of cellular hypertrophy and, to a lesser degree, cell replication (hyperplasia). Concomitantly, CRG comprises functional adaptations such as increases in glomerular filtration rate (GFR) and renal blood flow (RBF) [1, 2]. The CRG after Unx has been widely studied in rodents where increased cell number and size and enhanced activity of the excretory structures occur [3–5]. Several in vivo studies have demonstrated that renal tissue undergoing CRG would be less susceptible to injury caused by induction of acute renal failure (ARF) [6–11]. Thus, in a rat model of glycerol-induced ARF, Ramos et al. [6] determined that previous Unx enhanced the recovery of renal function, and suggested that hemodynamical changes during CRG, like an increase in RBF, accounted for the protection. Induction of CRG also attenuated functional and histological damage after ischemia-induced ARF [7, 8]. In this model, the protective effect of CRG was attributed to alterations in the renal milieu rather than to compensatory hypertrophy. Many of the environmental factors that are modified during CRG have been proposed to have beneficial effects against ischemic injury [9–11]; however, the precise mechanisms that lead to this protection, as well as their validity in other renal pathologies, remain to be investigated.

Hemolytic uremic syndrome (HUS) is a clinical entity associated with infection with enteropathogenic strains of *Escherichia coli.* The syndrome is characterized by the presence of hemolytic anemia, thrombocytopenia and renal insufficiency, and constitutes the main cause of ARF in childhood [12]. The *E. coli*- derived Shiga toxin (Stx) binds mainly to glomerular endothelial and tubular epithelial cells within the kidney, and directly inhibits protein synthesis [13, 14]. Endothelial cell injury and activation is thought to prompt a progression towards vascular thrombosis and platelet aggregation, in turn causing vascular obstruction and precipitating distal ischemia [15].

Among the various vasoactive substances that are modulated during HUS [16, 17], the alterations in the nitric oxide (NO) pathway are of crucial importance. This molecule is an endogenous vasodilator synthesized from L-arginine by a family of enzymes called NO synthases (NOS) [18, 19]. An upregulation of NO production, together with other anti-thrombogenic agents, has been described in patients coursing the acute phase of thrombotic microangiopathies [20]. Moreover, we have demonstrated that endogenously produced NO plays a protective role in the early phase of Stx-induced pathogenicity [21]. Nitric oxide has been implicated in mediating some of the hemodynamic adaptations to Unx in rats as well. Valdivielso et al. [22] and more recently Sigmon et al. [23] determined that NO blockade inhibits the increase in RBF following Unx. The former study also showed enhanced NO production in kidneys from Unx animals. In the kidney under physiological conditions, NO is mainly derived from the constitutive endothelial NOS (eNOS) and plays a homeostatic role, controlling normal glomerular and renal hemodynamics [24–26]. Conversely, expression of inducible NOS (iNOS) is hardly detected in normal kidney and it is induced in infiltrating and several types of resident cells under certain pathological conditions [27–29].

Based on these antecedents, this study was designed to test the hypothesis that the compensatory changes that take place during CRG could attenuate the toxicity of Stx on renal tissue. The particular involvement of the NO pathway was analyzed.

Using a mouse model of HUS induced by intravenous injection of Stx type 2 (Stx2) [30], it was determined that the CRG that follows Unx provided striking protection against Stx2-mediated renal damage and mortality. In order to explore the underlying mechanisms, some of the renal parameters usually modified by Unx or Stx2 were studied. These were: renal enlargement and cortical cell proliferation, intrarenal blood flow, and eNOS and iNOS expression. Results suggest that CRG induces protection of the kidney, which counterbalances the severe renal ischemia induced by Stx2, and this is mainly related to preserved renal blood flow and eNOS expression.

Materials and methods

Stx2 Pure Stx2 was provided by Dr. Junichi (Denka Seiken Co., Tokyo, Japan). Purity, endotoxin contamination and cytotoxic activity on Vero cells were tested previously [21, 30]. In the preliminary experiments, the in vivo lethality was evaluated by serial dilutions in pyrogen-free saline. The LD_{50} was 2.5×10^{-5} mg/kg body weight (~500 pg/mouse). In those experiments designed to see protection (Figs. 1 and 2), an intravenous dose of $2LD_{50}$ was chosen, for which the mean time to death was 3.5 days. In the other experiments, a single LD_{50} was injected.

Animals and unilateral nephrectomy Two month-old male BALB/c and C.C3H $Tlr4^{lps\text{-}d}$ mice (congenic for the defective LPS response allele from C3H/HeJ mice), weighing 20–25 g, and born and raised in the animal facilities of the Academia Nacional de Medicina, Bs. As., were used. All studies were conducted according to the principles set forth in the *Guide for the Care and Use of Laboratory Animals* from the NIH [31]. Animals were subjected to right unilateral nephrectomy through a lumbar incision under sodium pentobarbital anesthesia. Sham operation was performed by removing the kidney and then returning it to the renal fossa. Except where indicated, Unx was performed 48 hours before Stx2 injection.

Endogenous NO synthase (NOS) modulation Nitric oxide synthase was blocked with N^G-nitro-L-arginine methyl ester (L-NAME, Sigma Chemical Co, St. Louis, MO, USA). Mice were treated with a previously tested dose of L-NAME via their drinking water (1 mg/ml; mean dose of L-NAME: 5 mg/kg body wt/day) [21, 32]. L-NAME administration began 48 h before Unx (groups L-NAME+Unx and L-NAME+Unx+Stx2) or 48 h before Stx2 injection (group L-NAME+Stx2), and lasted for 15 days. On day 15, it was suspended in order to avoid severe systemic effects on blood pressure and body weight. Previous determinations [21] showed that L-NAME alone had no effect on mice survival or plasmatic urea values.

Serum determination of urea Blood samples were obtained by puncture of the retro-orbital plexus. Biochemical determinations of urea in mouse sera were performed in an autoanalyzer CCX spectrum (Abbot Diagnostics Systems, Buenos Aires, Argentina) following standardized instructions.

Assessment of weight Kidneys were weighed at multiple times after Unx. With L-NAME treatment ending at day 15, no significant changes in body weight occurred throughout the experimental period; for this reason, renal weight values were not corrected with body weight. To determine the dry weight, the kidneys were kept in an oven at 80°C until the dry weight was constant for three consecutive evaluations. The percentage increase was

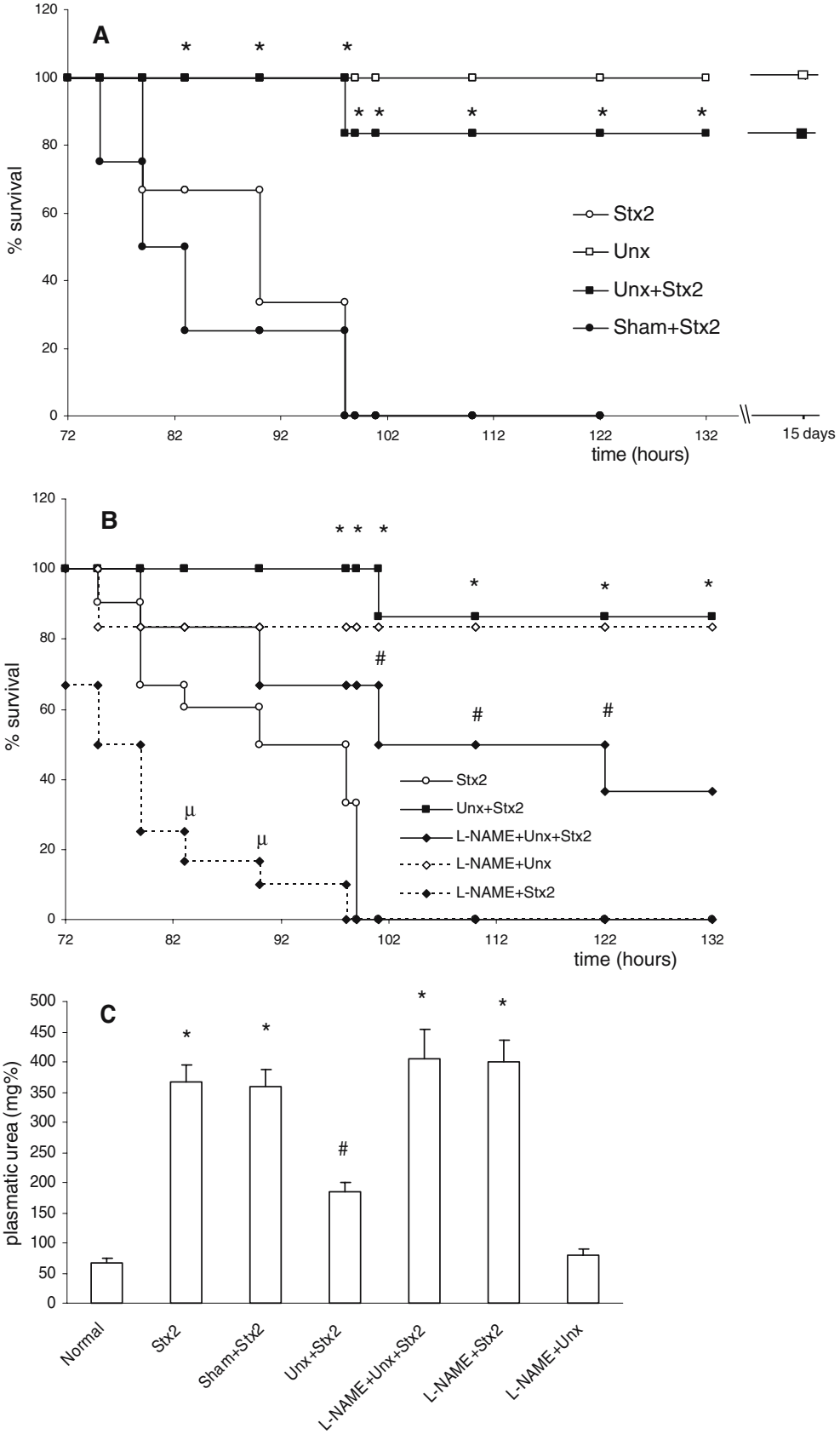
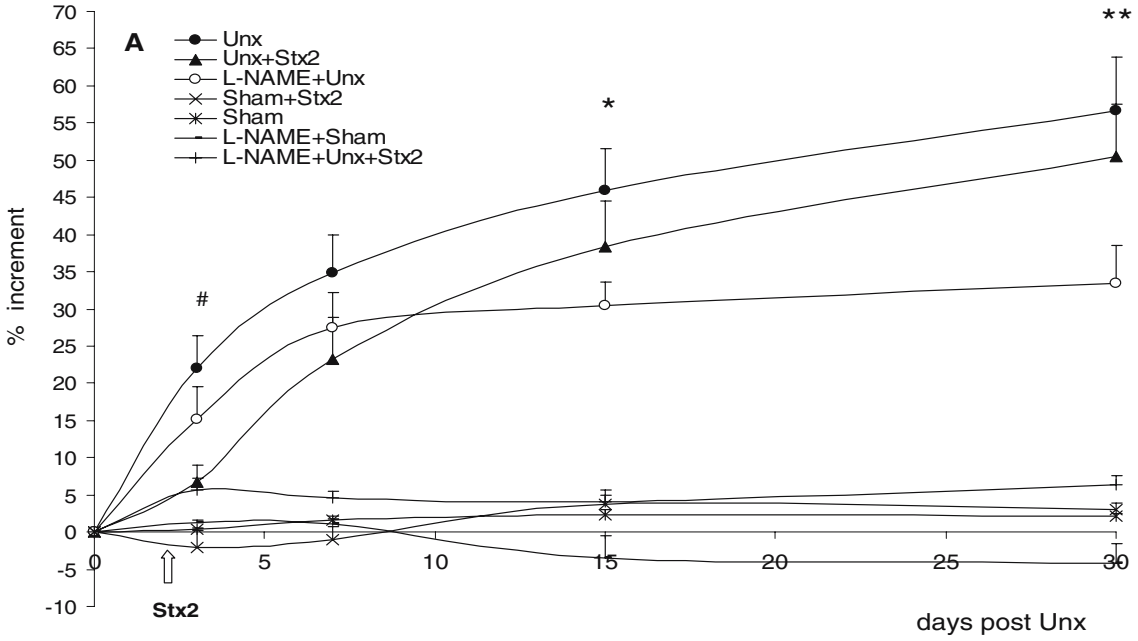


Fig. 1a–c *Effect of uninephrectomy upon Shiga toxin-induced lethality and renal damage*. **a** Mice were subjected to uninephrectomy (Unx+Stx2) or sham-operation (Sham+Stx2) 48 hours prior to the injection of a lethal dose of Stx2. Time of death is represented up to 132 hours after Stx2 injection; afterwards it remained constant up to the end of the experimental time (15 days). Survival percentages corresponding to 24 mice/experimental group are shown (*: $p<0.001$ versus Stx2 and Sham+Stx2). **b** Mice were treated with L-NAME from 48 hours before performing Unx (groups L-NAME+Unx+Stx2 and L-NAME+Unx) or 48 hours before Stx2 (group L-NAME+Stx2) up to the end of the experiment (day 15). Stx2 was injected 48 hours after surgery (groups Unx+Stx2 and L-NAME+Unx+Stx2). Survival percentages of 20–24 mice/experimental group are shown (*: $p<0.01$ versus L-NAME+Unx+Stx2; #: $p<0.01$ versus Stx2; μ: $p<0.001$ versus Stx2). **c** Plasma urea determinations were made three days after Stx2 injection. Data show mean±SE of plasmatic urea values (mg%) from 12 mice per group (*: $p<0.0001$ versus normal; #: $p<0.001$ versus Stx2 and Sham+Stx2)



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Days after Unx	Unx n=8	Unx+Stx2 n=8	Sham +Stx2 n=6	L-NAME +Unx n=8	Sham n=8	L-NAME +Sham n=6	L-NAME +Unx+Stx2 n=6
3	$22.1\pm3.8^{\alpha}$	10.7±2.2	12.7±2.6	14.2±3.7	12.4±1.6	12.2±4.1	12.6±2.1
7	$25.4\pm2.3^{\beta}$	18.4±3.2	10.5±5.2	14.1±2.8	13.4±2.0	11.8±5.0	11.3±3.9
15	$34.6\pm3.8^{\gamma}$	$29.3\pm5.6^{\Delta}$	14.2±6.6	$21.2\pm4.5^{\pi}$	12.9±4.3	10.1±4.7	14.1±5.4
30	$38.7\pm4.4^{\gamma}$	$33.0\pm6.1^{\Delta}$	14.0±4.2	$23.5\pm2.9^{\Delta}$	14.3±4.4	6.2±2.4	14.2±5.0

Fig. 2a,b *Increase in dry weight and protein content of the kidney remaining after uninephrectomy.* The remanent kidney was excised at multiple time points after Unx; the percentage increase in renal dry weight (**a**) and the total protein content in mg (**b**) were obtained. Values are expressed as mean±SE of *n* mice per group (**a**: #: $p<0.01$ versus Unx+Stx2; *: $p<0.05$ and **: $p<0.001$ versus L-NAME+Unx, $n=12$; **b**: α: $p<0.05$ versus every other group except L-NAME+Unx; β: $p<0.01$ versus all groups except Unx+Stx2; γ: $p<0.05$ versus all groups except Unx+Stx; π: $p<0.01$ and Δ: $p<0.001$ versus L-NAME+Unx+Stx2)

calculated by comparing the weight of the remaining (left) to that of the excised (right) kidney of the same animal.

Determination of protein content Protein concentration was determined by the method of Bradford [33] using bovine serum albumin as a standard.

Cell cycle analysis Cell cycle analysis was performed by flow cytometry [5]. Briefly, the kidneys were longitudinally bisected and sharply divided at the corticomedullary junction under a magnifying glass. Samples were limited to the cortex, since the proliferative response during CRG takes place mainly in this cell population [1, 3]. Tissue was mechanically minced, and the cell pellet was suspended in a solution containing propidium iodide (50 μg/ml in 10^{-2} M Tris buffer) with 5 mM $MgCl_2$ and 1 mg/ml sodium azide, and incubated for 24 h at 4°C. RNA contaminants were eliminated with ribonuclease (10 μl of a 6 mg/ml RNase per 1 ml of suspension). The fluorescence was measured using a FACScan flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA). The percentage of cells in each phase was analyzed by a Modfit LT 2.0 program.

Renal flow studies Unilateral renal flow was determined by means of gamma chamber renography, from the relative uptake of ^{99m}Tc-DMSA-Sn (Bacon Laboratorios, Bs. As., Argentina) [34, 35]. Images were taken five to ten

minutes after intravenous injection of the tracer, in anesthetized mice at multiple time points following Unx or Stx2 administration. Counts per area (cpa) corresponding to the cortical and medullary areas were obtained by making up an oval gate comprising the darker, central area, which represents medullary circulation, and subtracting counts within it from counts of the whole kidney. Sham-operated animals, whose renal blood flow did not differ from normal intact mice (data not shown), were used as controls.

Immunohistochemistry for eNOS and iNOS Paraffin-embedded kidney sections were used. Indirect immunoperoxidase staining was performed using rabbit-raised polyclonal antibodies against eNOS (1:200) and iNOS (1:100) (Cat N° 160880 and 160862, respectively, Cayman Laboratories, Ann Arbor, MI, USA). Incubation with the primary antibody was performed overnight at room temperature. The second incubation was done with the biotinylated antirabbit immunoglobulin serum for 30 minutes. Sections were then incubated with the streptavidin-peroxidase complex (Dako LSAB2–System HRP–DAB; Dako Corp., Carpinteria, CA, USA) and counterstained with hematoxylin. Sections of mouse salivary gland were used as a positive control for iNOS [36].

Statistics For survival data, percentages were tested for significance using the Fisher's exact test. All other data correspond to the mean±SE of individual mice. Statistical differences were determined using one-way analysis of variance (ANOVA). Comparisons between two groups were performed using unpaired Student's *t*-tests.

Results

Stx2 toxicity during compensatory renal growth: nitric oxide involvement

Mice were subjected to Unx in order to induce CRG and 48 hours later, when according to preliminary observations CRG was already initiated, they were injected with a lethal dose of Stx2. Survival data shown in Fig. 1a indicate that uninephrectomy provides considerable (almost complete) protection against Stx2-induced mortality. Although the graph shows survival percentages for 132 hours after Unx, uninephrectomized mice continued to live and did not show signs of renal damage for an additional six months (not shown). Moreover, Unx mice were still protected when the toxin was administered up to one month after Unx (Table 1).

Table 1 Protection at time points after Unx

Stx2 injection	Unx+Stx2	Sham+Stx2	Stx2
day 15 post surgery	90.9±10.3**	12.8±3.4	20.0±5.6
day 30 post surgery	91.6±6.7**	8.3±2.4	6.2±3.1

The table shows the mean±SEM values from three separate experiments, where at least 12 mice per experiment were exposed to a lethal dose of Stx2, 15 or 30 days after Unx (Unx+Stx2) or sham operation (Sham+Stx2); intact mice (Stx2) were used as a control. Survival percentages were obtained 100 hours later (**: $p<0.0001$ versus Sham+Stx2 or Stx2; $n=3$)

To determine the relationship between NO and CRG-mediated protection, Stx2 lethality was assessed in Unx mice where NO production had been inhibited by L-NAME during CRG progression (Fig. 1b). The high protective effect of CRG upon Stx2-induced mortality (Unx+Stx2) was partially impaired by L-NAME administration (L-NAME+Unx+Stx2). As previously reported [21], NO inhibition had a direct worsening effect upon Stx2-mediated mortality (L-NAME+Stx2); it is worth noting that CRG was also able to attenuate the enhancing effect of L-NAME.

In this HUS model, toxin-induced renal failure is evidenced by an early rise in plasma urea [30]. Renal toxicity caused by Stx2 during CRG was then measured as the increase in urea concentration in the plasma of Unx mice treated with Stx2 (Fig. 1c). In line with the survival data, Unx before Stx2 injection reduced the severity of the resultant increase in uremia. At day 3 after Stx2 injection, the intact (Stx2) or sham-operated (Sham+Stx2) mice showed elevated uremia, denoting severe renal damage, while in Unx mice (Unx+Stx2) urea levels were significantly lower. Administration of L-NAME throughout CRG (L-NAME+Unx+Stx2) abolished the attenuating effect of Unx. In Unx mice (Unx+Stx2) and in the surviving mice from the L-NAME+Unx+Stx2 group, plasmatic urea returned to normal values at day 7 after Stx2 injection (by which time most of the other Stx2-treated animals had already died), and remained normal until at least day 20 (day 7: 72.1±6.9 and 68.6±7.1; day 20: 65.7±8.8 and 73.7±6.1 mg urea %, for the Unx+Stx2 and L-NAME+Unx+Stx2 groups respectively, not significantly different from control mice; $n=12$). Uninephrectomy operation alone did not alter plasmatic urea levels throughout the experimental period (not shown).

Similar toxic effects in the kidney have been ascribed to LPS, although at considerably higher concentrations [30, 37]. In spite of the insignificant amount of LPS detected in the Stx2 employed herein (less than 40 pg/μg Stx2, i.e., $2x10^{-8}$ μg LPS/mouse), a control experiment was performed where Stx2 was injected into both normal BALB/c and C.C3H *Tlr4*$^{lps\text{-}d}$ mice (unresponsive to LPS). Comparable increases in plasmatic urea (320.6±23.1 and 351.2±16.7 mg% for control and C.C3H *Tlr4*$^{lps\text{-}d}$ mice, $n=3$, not significant) were found 72 hours after Stx2 injection, indicating that the effects observed on the kidney were actually mediated by the toxin.

Effect of Stx2 and NO on renal growth and perfusion during CRG

To further study the pathogenicity of Stx2 during CRG, we analyzed its effect on two characteristic adaptative changes: enhanced renal growth and perfusion. The importance of NO availability was also analyzed. Control animals or animals pretreated with L-NAME were subjected to Unx,

and a subset was injected with Stx2 48 hours later. Renal dry weight was determined at various intervals after surgery (Fig. 2a). Uninephrectomy was followed by a progressive increase in renal dry weight. Shiga toxin 2 alone (group Sham+Stx2) did not alter renal weight, neither in the dying nor in the surviving mice. During CRG, Stx2 administration (group Unx+Stx2) slowed the Unx-induced renal weight gain for the first 1–3 days, whereas L-NAME (group L-NAME+Unx) significantly prevented it. L-NAME-mediated effects became statistically significant at day 15 and persisted until at least day 30, although L-NAME was removed earlier (day 15). Administration of L-NAME and Stx2 to Unx mice (group L-NAME+Unx+Stx2) resulted in an additive inhibitory effect of both treatments. Finally, no significant variations in renal dry weight were seen in control sham-operated mice pretreated or not with L-NAME (Sham or L-NAME+Sham). Renal weight correlated with the protein content of the remaining kidneys (Fig. 2b).

Proliferation rates of cells from the cortex of the remaining kidneys indicated that Unx was followed by DNA synthesis and replication, as evidenced by the increase in the number of cells entering both the S and G_2M phases (Fig. 3), and the decrease in the percentage of quiescent (G_0G_1) cells (not shown). A significant increase in cell replication was seen three days after Unx, with the peak proliferation occurring at day 7. An early significant increase in proliferation was also seen in the control sham-operated mice, but this response rapidly declined. Stx2 administration per se did not alter basal proliferation, but delayed the proliferative response triggered by Unx (group Unx+Stx2 vs Unx) and prevented proliferation after sham operation (group Sham+Stx2 vs Sham). L-NAME administration (group L-NAME+Unx) also inhibited proliferation during CRG, whereas the combination of Stx2 and L-NAME (group L-NAME+Unx+Stx2) completely abolished it.

Renal blood flow (RBF) was studied by means of a gamma chamber. Measurement of the total RBF (Fig. 4) showed that Unx induced a progressive increase, which was blocked by L-NAME pretreatment. Administration of L-NAME to intact mice also decreased RBF; this fact has been documented and was attributed to the pressor effect of the drug [22, 24, 25]. On the other hand, Stx2 induced a severe drop in renal perfusion; performing the Unx 48 hours before Stx2 administration prevented this ischemic effect, restoring the renal flow to 80% of its normal value. Uninephrectomy was not able to restore Stx2-mediated inhibition when L-NAME was present.

The discrimination of the partial contribution of the cortical and medullary flows to the total renal flow is shown in Fig. 5. The great increase in RBF during CRG was due to rises in both the cortical and medullary compartments. Shiga toxin 2 strongly reduced cortical and medullary flow in sham-operated mice, while in Unx animals the toxin only affected the flow within the medulla. In these animals, Stx2 reversed the Unx-induced enhancement of medullary flow and reduced it to approximately half of its control value; however, the toxin was unable to decrease the Unx-mediated increase in cortical flow.

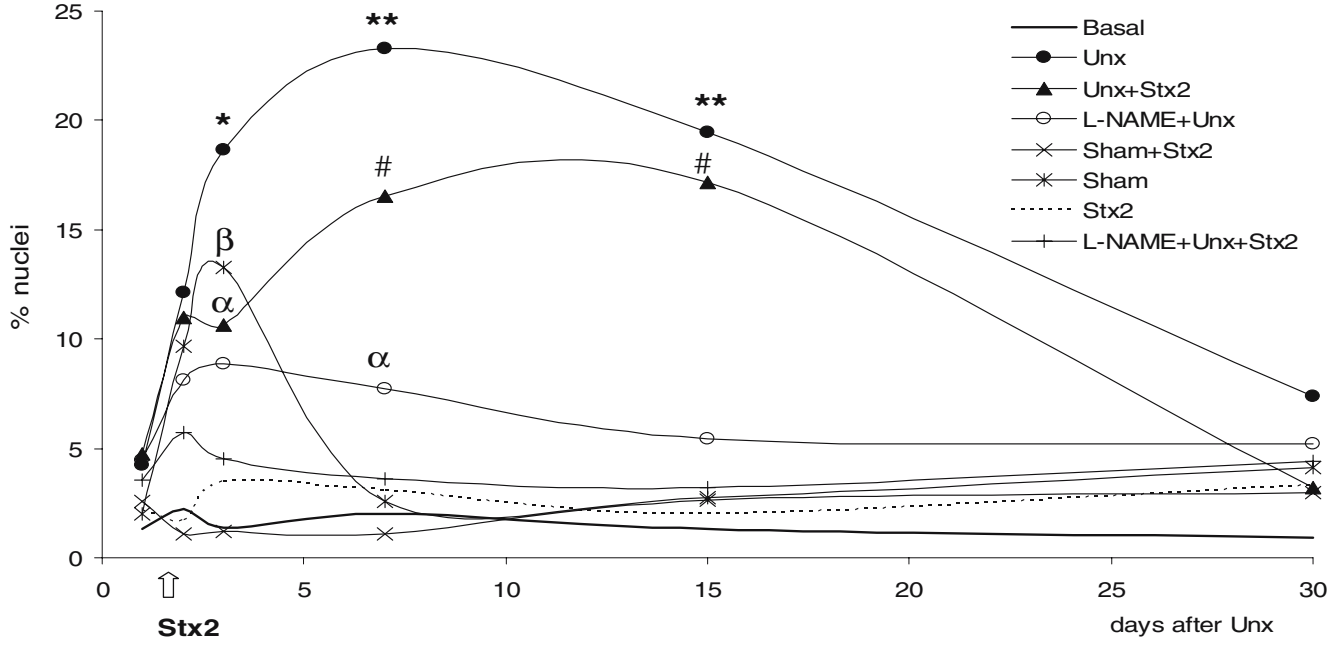


Fig. 3 *Percentage of cells in replication (S+G2M) in the cortex of the contralateral kidney following Unx*. The data corresponds to one representative experiment out of three, in which six mice per group were used for each time point (*: $p<0.01$ versus basal and $p<0.05$ versus Unx+Stx2 and L-NAME+Unx; **: $p<0.001$ versus basal, Sham and L-NAME+Unx; #: $p<0.05$ versus basal, L-NAME+Unx, Stx2, Sham+Stx2 and L-NAME+Unx+Stx2; α: $p<0.05$ versus L-NAME+Unx+Stx2; β: $p<0.05$ versus basal and Sham+Stx2)

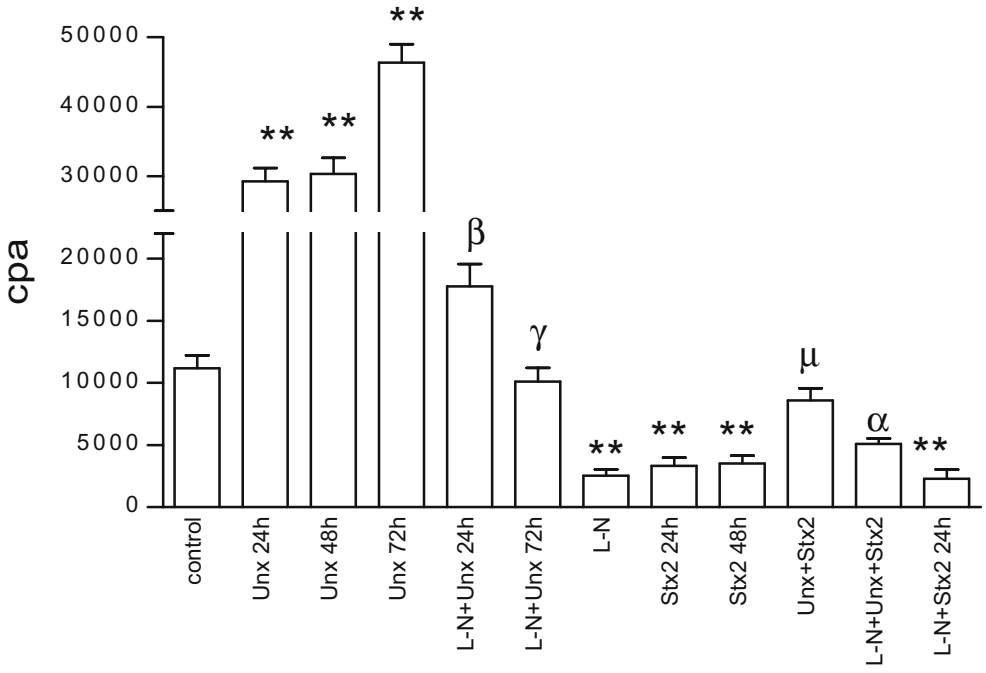


Fig. 4 *Effect of Unx, L-NAME and Stx2 on total renal blood flow (RBF).* Counts within the total renal area (cpa) were obtained at different time points after Unx or Stx2 injection. In the Unx+Stx2 and L-NAME+ Unx+Stx2 groups, Stx2 was injected 48 h after Unx, and RBF was obtained 24 h later. One representative experiment of a series of three is shown (**: $p<0.001$ versus control sham-operated animals; β: $p<0.01$ versus Unx 24 h; γ: $p<0.001$ versus Unx 24, 48 and 72 h; μ: NS versus controls and $p<0.01$ versus Stx2; α: NS versus Stx2; $p<0.01$ versus controls, Unx+ Stx2 and L-NAME and $p<0.05$ versus L-NAME+Stx2; $n=4$)

Renal expression of endothelial and inducible NO synthases

The data above suggest that NO is an important mediator during CRG and CRG-mediated protection. These observations prompted us to study the modulatory effect of Unx and Stx2 on the NOS intrarenal expression. A basal expression of eNOS was observed in kidneys from normal animals (Fig. 6a), with no detectable expression of iNOS (not shown). Uninephrectomy alone induced a progressive and time-dependent increase in eNOS (Fig. 6b), with no effect on iNOS expression (Fig. 6b insert). In the Stx2-treated group, basal eNOS expression either decreased or disappeared (Fig. 6c), while staining for iNOS became detectable (Fig. 6d). In Unx mice, although they were treated with Stx2, eNOS expression was preserved (Fig. 6e) with little or no iNOS expression (Fig. 6f). Figure 6g summarizes the immunohistochemical findings.

Counterstaining of the slides allowed us to see that the basal expression of eNOS was confined to glomerular loops and to a lesser degree to the peritubular vasculature, whereas Unx- induced eNOS was additionally detected in tubular and glomerular epithelia. Staining for iNOS was localized in capillaries, epithelial and mesangial cells within the glomerulus and epithelial tubular cells. Strongly positive mononuclear stromal or infiltrating cells were also present in the interstices.

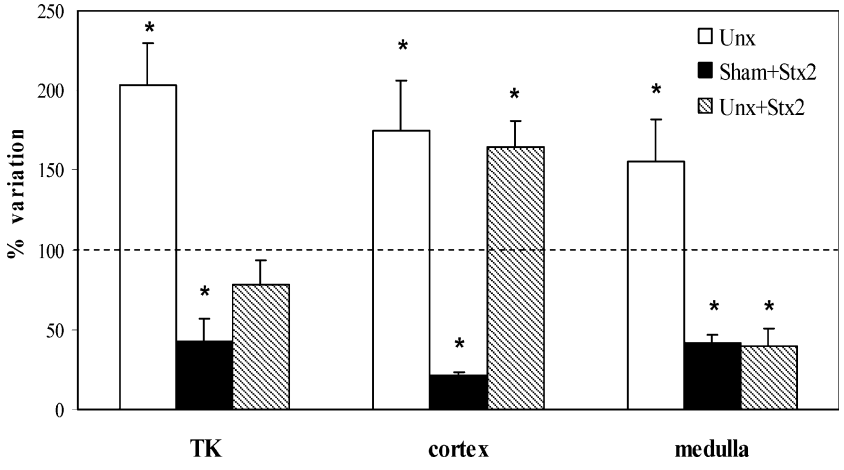
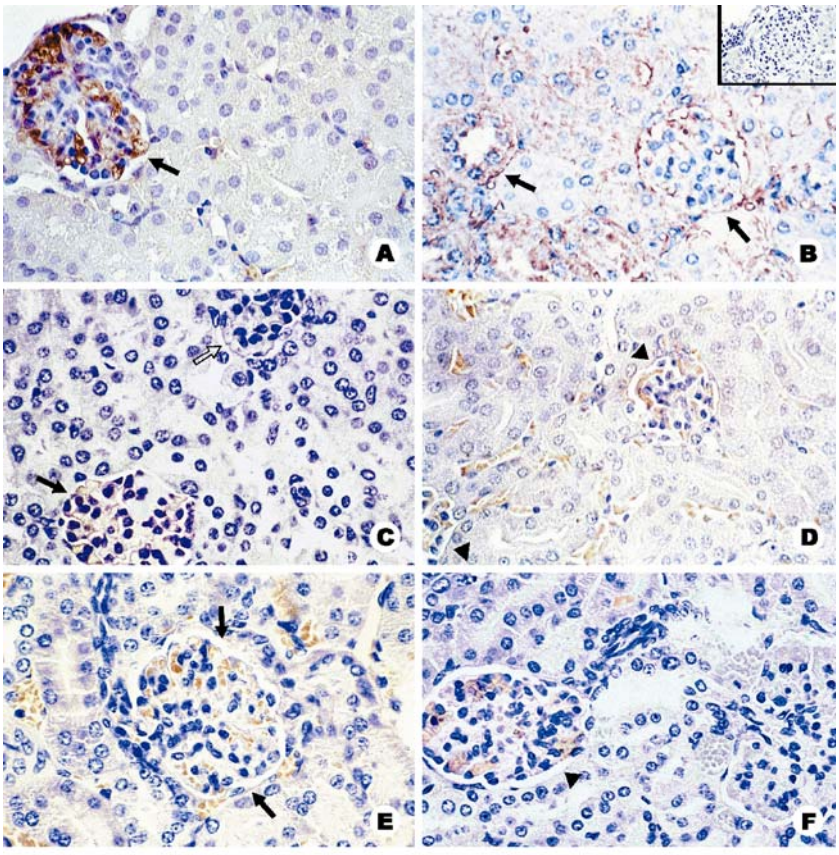


Fig. 5 *Effect of Stx2 and Unx on cortical and medullary blood flow.* Stx2 was injected 48 h after operation (groups Sham+Stx2 and Unx+Stx2). Twenty-four hours later, the counts per area (cpa) corresponding to the total kidney (TK) were obtained, and the partial cortical and medullary flows were estimated by means of a gate. In the figure, *bars* represent the percentage change in blood flow with respect to control sham-operated animals, which was taken to be 100%. Statistical analysis was performed on cpa values (*: $p<0.01$ significantly different from controls, $n=8$)



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Treatment	eNOS	iNOS
Control	+	-
Sham	+	-
Unx 24h	++	-
Unx 48h	+++	-
Unx 72h	++++	-
Stx2 24h	+/-	+
Stx2 48 to72h	+/- to -	+/++
Sham + Stx2 72h	-	++
Unx 48h + Stx2 24h	+	-
Unx 48 h + Stx2 48h	+	+/-

Fig. 6 *Immunohistochemistry for eNOS and iNOS in renal sections of normal, Unx, and Stx2- treated mice (40×).* **a** Staining for eNOS in normal kidneys. **b** Staining for eNOS and **b insert** staining for iNOS in kidney from nephrectomized mouse, 72 h after Unx. **c** Staining for eNOS and **d** staining for iNOS 48 hours after the injection of 500 pg Stx2. **e** Staining for eNOS and **f** staining for iNOS in kidney from Unx+Stx2 mouse, 72 h after Unx and 48 h after Stx2 (500 pg). (**g**) Presence of positive staining for NOS, graded from − to ++++ according to the number of positive structures and the intensity of the staining. Twenty 40× fields were analyzed

As typical Stx2-induced renal injury is focal, and zones of glomerular or tubular alterations are detected among well-preserved structures [21], it was possible to see that eNOS expression was generally associated with renal areas of preserved integrity: round lobulated glomeruli with Bowman's space and normal mesangium; glomerular capillaries lined by dark-stained endothelium; typical tubular epithelium (Fig. 6; filled arrows). In contrast, eNOS was low in retracted, hypercellular injured glomerulus (Fig. 6; empty arrows). Inducible NOS expression occurred mainly in damaged glomeruli, areas of mesangial proliferation and occasionally within cytoplasm of altered tubular epithelial cells (Fig. 6; arrowheads), but was absent in areas of mild or no damage.

Discussion

The lack of an animal model which reflects all features of human HUS is possibly related to interspecies differences in the expression of the Stx-specific receptor, the globotriaosylceramide (GB3). In spite of the fact that the GB3 receptor is absent in the glomeruli of mice, the mouse model of HUS, by systemic injection of Stx2, reproduces the acute renal failure lesions, mainly by inducing tubular necrosis. Glomerular alterations, probably arising as a consequence of tubular damage or cytokines released in the kidney, are observed as well, along with other characteristic systemic alterations (platelet activation, thrombocytopenia, neutrophilia) [21, 30].

The aim of this work was to investigate whether Stx2 toxicity could be attenuated by altering the basal state of the kidney through the stimulation of CRG. We found that three of the pathogenic effects of Stx2—renal injury, decreased perfusion of the kidney and mortality—were lowered by previous induction of CRG with Unx. The abolition of NO production by systemic administration of L-NAME partially prevented the protective effect of CRG, suggesting that it is mediated in part by NO. We have previously reported data indicating that NO exerts a

protective role against Stx2 toxicity [21]. In addition, NO availability could be required for the compensatory changes during CRG. In this sense, a work from Valdivielso et al. [22] suggested that NO is an important mediator in the hemodynamic adaptation that follows Unx. We found that NO is necessary not only for the increase in RBF during CRG but also for the hypertrophic and hyperplastic responses. While the blockade of Unx-induced increase in RBF by L-NAME was evident from 24 hours post Unx and beyond, inhibition of proliferation, protein content and weight increases were detected afterwards (3, 7 and 15 days after Unx respectively). This suggests that the last responses are a delayed consequence of a deficient hemodynamic adaptation, which in turn could be due to the lack of NO. This hypothesis reinforces Sigmon's, who in a recent report [23] suggested that NO-induced vasodilation is the early stimulus that precedes hypertrophy in CRG.

Shiga toxin 2 also interfered with the hypertrophic and hyperplastic responses to Unx by producing a delay in the first 5–7 days. However, we did not detect a direct effect of Stx2 on the basal size and cell proliferation of the kidney (Figs. 2 and 3). It has been proposed that dividing cells are more susceptible to Stx than quiescent cells [38, 39]; in this case, the direct cytotoxic action of Stx2 on target cells is likely to be amplified in conditions of intense growth like CRG. Despite this, the fact that mice are less susceptible to Stx2 during CRG led us to propose that systemic factors other than the direct specific cytotoxicity, probably related to ischemia, are involved in Stx2-induced renal damage and death.

Renographic studies revealed that Stx2 provoked a severe drop in the blood flow of the kidney, which agrees with Stx-induced renal alterations comprising ischemic injury and varying degrees of vascular occlusion found both in humans [40] and in mice [21]. The increase in renal perfusion during CRG was able to counterbalance the ischemic effect of Stx2. Nitric oxide inhibition would blunt the increase in renal flow, thus reducing the protection afforded by Unx.

In Unx mice treated with Stx2, renal flow was close to 80% of the normal and CRG occurred almost completely; in this group, Stx2 induced a very low rate of mortality. In Unx mice treated with L-NAME and Stx2, an additive inhibition of the Unx-induced increase in RBF was seen. The remaining flow in this group was apparently enough to confer a degree of protection (~50% of the animals survived), while it was not sufficient to sustain CRG (no renal growth or proliferation were observed). These results suggest that the maintenance of renal perfusion in the first hours, rather than renal growth, would account for the protection during CRG.

The distinction between cortical and medullary flow made it possible to conceive that after Unx both regions showed enhanced blood flow. Shiga toxin, on the other hand, decreased the perfusion in both compartments as well. The observation that performing Unx prevented Stx2-mediated flow reduction only in the cortex, not in the medulla, was of special interest. It suggests that the medullary flow is a major target of Stx2 in Unx mice, and that the maintenance of renal blood flow in these animals is based mostly on the cortical flow. Although further demonstration by a more accurate technique will be necessary to check these ideas, much speculation can be made based on them. The fact that cortical and medullary flows respond to different regulatory pathways constitutes a possible explanation. While cortical blood flow and the consequent pressure of filtration are highly dependent upon the systemic volemia and the cardiac output [15], both parameters augmented during CRG [4], in the medulla, other factors like hormonal balance could be regulating its function [15, 41]. Nevertheless, since Unx individuals continue to live and do not show signs of renal insufficiency, medullary flow may presumably be restored after the observation time employed in our experiments. In this sense, there is consensus about the fact that when postischemic restitution of blood flow occurs, it is not homogeneous in the different regions within the kidney [42–44].

Kidneys exposed to Stx2 showed elevated levels of iNOS expression, whereas eNOS expression decreased. Within glomeruli such variations are not likely to be due to direct Stx2-mediated action, but to systemic alterations like the widespread thrombosis and decreased renal flow resulting from hemodynamic imbalance. An opposite regulation of eNOS and iNOS was previously documented in certain renal pathologies [28, 29, 45], as well as in a model of ricin-induced HUS [46]. Compensatory renal growth tended to neutralize Stx2 effects on both enzymes. In the first case, eNOS expression was preserved from Stx2 inhibition. This is likely to be due to increased RBF following Unx, which should directly activate constitutive eNOS by increasing shear stress, as previously suggested [22, 23]. In addition, the proliferative response during CRG could be increasing the number of sites for the expression of eNOS. In the second case, iNOS induction by Stx2 did not take place during CRG. As iNOS appeared to be confined to areas of tissue injury, the preservation of kidney integrity in Unx mice could also account for this fact. In a previous work we demonstrated that the inhibition of NO production by L-NAME (an unselective NOS inhibitor) strongly worsened Stx2 toxicity, whereas the highly selective iNOS inhibitor L-NIL had no effect [21]. This observation led us to postulate that inhibition of eNOS activity, but not activation of iNOS, is the most relevant mechanism in the pathogenicity of Stx2. The induction of iNOS by Stx2 would constitute a late consequence of injury and inflammation. In this context, we propose that the protection observed during CRG is pre-eminently due to the conservation of eNOS activity rather than to the blockade of iNOS.

In contrast to our results, the study from Valdivielso et al. [22] demonstrated that there were no differences in eNOS but a higher amount of iNOS and activity in glomeruli from Unx compared with sham-operated rats. Those results were obtained from a glomerular cell culture, where tubular epithelial and peritubular vascular cells were excluded. As the precise contribution of each cell type within the cortex

to the expression of eNOS and iNOS is not known, differences may be due to the relative abundance of the various cell populations. Additionally, other environmental modulatory factors may be operating in our in vivo model of HUS.

This work was performed with a view to further elucidating the pathogenic mechanisms of Shiga toxin in order to find new therapeutic tools for mitigating renal damage during HUS. Although unilateral nephrectomy is an invasive ablative procedure, not applicable to the human situation, the finding that enhanced growth and activity of the kidney could provide protection is of interest in the search for strategies that may be used to counteract Stx2 pathogenicity. In this respect, we are currently evaluating whether plasmatic expansion (a clinical situation analogous to uninephrectomy) or serum from uninephrectomized animals may also protect mice exposed to Stx2. Moreover, while this manuscript was being written, a work was published [47] that reported that intravenous volume expansion provides nephroprotection during *E. coli* O157:H7 infection in children. It is concluded from the present study that changes during CRG confer a high resistance to the effects of Stx2. Two of the mechanisms involved in this protection are the exacerbation of the basal conditions in the kidney, in particular those of renal blood flow, and the preservation of renal eNOS expression from its inhibition by Stx2.

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