BIOCELL
2010, 34(3): 121-132

ISSN 0327 -9545
PRINTED IN ARGENTINA

WT-1 mRNA expression is modulated by nitric oxide availability and Hsp70 interaction after neonatal unilateral ureteral obstruction

LUCIANA MAZZEI[1], ISABEL MERCEDES GARCÍA[1], VALERIA CACCIAMANI[1], MARÍA EUGENIA BENARDÓN[1] AND WALTER MANUCHA[1, 2, 3]

1. Área de Fisiopatología, Departamento de Patología. Facultad de Ciencias Médicas. Universidad Nacional de Cuyo. Mendoza.
2. IMBECU-CONICET (National Council of Scientific and Technical research of Argentina)
3. Área de Farmacología, Departamento de Patología. Facultad de Ciencias Médicas. Universidad Nacional de Cuyo. Mendoza.

Key words: obstructive nephropathy, apoptosis, statins, kidney development

ABSTRACT: Wilms tumor gene 1 (*wt-1*), a key regulator of mesenchymal-epithelial transformation, is downregulated during congenital obstructive nephropathy, leading to apoptosis. There is a functional interaction between WT-1 and inducible nitric oxide synthase (iNOS). In this regard, we reported that after neonatal unilateral ureteral obstruction, rosuvastatin prevents apoptosis through an increase in nitric oxide bioavailability, which in turn is linked to higher Hsp70 expression. Hence, the goal of this study was to determine whether a nitric oxide/Hsp70 interaction is involved in changes in WT-1 mRNA expression after ureteral obstruction. Neonatal rats submitted to experimental ureteral obstruction were treated with either vehicle or rosuvastatin for 14 days. Decreased nitric oxide and iNOS/Hsp70 expression associated with WT-1 low expression was shown in obstructed kidneys. Apoptosis was induced and it was associated with an increased Bax/BcL_2 ratio. Conversely, iNOS/Hsp70 upregulation and an increased WT-1 mRNA expression, without an apoptotic response, were observed in the cortex of obstructed kidneys of rosuvastatin-treated rats. Nitric oxide also modulated Hsp70 and WT-1 mRNA expression in MDCK cells. Finally, *in vivo* experiments with nitric oxide modulators support our hypothesis that WT-1 mRNA expression is associated with nitric oxide level. Results suggest that rosuvastatin may modulate WT-1 mRNA expression through renal nitric oxide bioavailability, preventing neonatal obstruction-induced apoptosis associated with Hsp70 interaction.

Introduction

Although congenital obstruction is the primary cause of end-stage renal disease in children, the mechanisms underlying chronic obstructive nephropathy have not yet been completely elucidated. Relating changes in gene expression to phenotypic patterns in human congenital obstructive nephropathy represent an advance in the identification of the genes/proteins that play important roles (Trnka *et al*., 2010). Wilms tumor gene (*wt-1*) identified as missing or mutated in embryonic kidney cancer cells (Buckler *et al*., 1991), appears to be the main determinant for initiating epithelial mesenchymal transformation. *In situ* hybridization studies have shown that *wt-1* is selectively expressed in the metanephric blastema and glomerular epithelium during embryonic and fetal development (Pritchard-Jones *et al*., 1990), suggesting that *wt-1* is involved in regulat-

*Address correspondence to: Walter MANUCHA.
Area de Fisiología Patológica, Depto. de Patología, Facultad de Ciencias Médicas, UNCuyo, 5500 Mendoza, Argentina.
Fax: (+54-261) 4135000 int. 2621.
E-mail: *wmanucha@fcm.uncu.edu.ar*
Received: July 13, 2010. Revised version received: November 20, 2010. Acepted: November 29, 2010.

ing proliferation and cell differentiation. Changes in *wt-1* expression pattern during ontogenesis suggest a significant role both during embryonic, as well as during fetal and postnatal, urogenital development.

Studies in neonatal rats may provide insight into the functional development of the kidney, since nephrogenesis continues at a rapid pace up to day 8 after birth and is virtually complete by days 14-19. In this regard, experimentally induced unilateral ureteral obstruction has emerged as an interesting model for studying neonatal hydronephrosis and for the assessment of potential therapeutic approaches. This model mimics, in an accelerated manner, the different stages of human neonatal hydronephrosis leading to tubulointerstitial fibrosis, apoptosis, epithelial-mesenchymal transition and tubular atrophy. Thus, unilateral ureteral obstruction in neonatal rats impairs nephrogenesis, glomerular maturation, and tubular cellular proliferation (Chevalier, 1998). The younger the age unilateral ureteral obstruction is performed, the more severe is the growth impairment of the ipsilateral kidney (Chevalier *et al*., 2002).

Unilateral ureteral obstruction results in altered cell proliferation and apoptosis in the neonatal rat kidney. These processes also occur in the developing obstructed human kidney. They are mediated by complex signaling pathways, including the heat shock protein response associated with nitric oxide (NO) interaction (Chan *et al.,* 2001). Many signals may positively or negatively affect the rat kidney after unilateral ureteral obstruction by altering regulatory proteins that initiate apoptosis and inducing changes in mitochondrial function (Manucha, 2007). Nitric oxide is able to either induce or inhibit apoptosis in different circumstances (Kim *et al.,* 1999), while factors such as BcL_2 have an anti-apoptotic effect. We aimed at characterizing alterations of the major modulators of mitochondrial apoptotic factors induced by neonatal unilateral ureteral obstruction in the rat kidney. Furthermore, we studied the effect of rosuvastatin treatment on animals subjected to unilateral ureteral obstruction, since previous studies have shown rosuvastatin protection against podocyte apoptosis *in vitro* (Cormack-Aboud *et al.,* 2009).

WT-1 is decreased in obstructive nephropathy, and this decrease promotes apoptosis. Obstructive nephropathy is also associated with down expression of genes related to renal vascular development as renin and angiotensin II AT_2 receptor (Liapis, 2003). We have shown that selective blockade of angiotensin II AT_1 receptor decreases renal interstitial fibrosis in unilateral ureteral obstruction, and this effect is associated with inducible nitric oxide synthase (iNOS) activity and expression (Manucha *et al*., 2004) as well as with Hsp70 expression (Manucha *et al*., 2005). In addition, Hsp70 is involved in cellular signaling pathways related to apoptosis, protein folding, and membrane translocation and in modulating the activity of tumor suppressor proteins, including p53 and WT-1 (Maheswaran *et al*., 1998). Johannesen *et al*. (2003) have shown functional interactions between the gene promoter of the main source of nitric oxide (iNOS) and WT-1, and a modulatory role of nitric oxide in the proliferation of T cells expressing WT-1 has been suggested (Marcet-Palacios *et al*., 2007). Interestingly, a cytoprotective role of nitric oxide associated with Hsp70 expression in neonatal hydronephrosis was recently shown (Manucha and Vallés, 2008).

A 3-hydroxy-3-methylglutaryl-coenzyme A reductase inhibitor, rosuvastatin, has a protective effect against podocyte apoptosis *in vitro* (Cormack-Aboud *et al.,* 2009). Renoprotection by statins has been recently associated with increased nitric oxide availability (Zhou *et al*., 2008). Previously, nitric oxide was thought to participate in tissue-specific cell maturation (Vasil´eva *et al*., 1997). However there is a paucity of information concerning possible interactions between nitric oxide and expression of genes linked to kidney development and function, like *wt-1*.

Therefore, the purpose of this work was to evaluate *wt-1* expression associated with nitric oxide bioavailability and Hsp70 interaction in apoptosis induction, using the neonatal unilateral ureteral obstruction model and renal epithelial cell cultures. Additionally, we aimed at characterizing alterations of the major modulators of mitochondrial apoptotic factors induced by neonatal unilateral ureteral obstruction, and studied the effect of rosuvastatin on this condition.

Materials and Methods

All experimental procedures were previously approved by the Ethical Committee on Laboratory Animal Research at the Cuyo University School of Medicine, Mendoza (IACUL A5780-01), in accordance with guidelines of the CEEA (Committee of Ethical Animal Experimentation of Argentina).

Animal's surgery and rosuvastatin treatment

Wistar Kyoto male and female rats were submitted to sham operation or complete unilateral ureteral obstruction within 48 hours after birth. Under isoflurane

anesthesia, the abdomen was surgically opened by a left lateral incision, the left ureter was exposed and ligated with a 6.0 silk suture. The incision was closed in a single layer. Pups recovered on a warmed surface and were returned to their mothers. All neonatal rats were treated once daily via gavage by means of a 24-gauge x 2-cm catheter (needle discarded) with either vehicle or 10 mg/kg/day rosuvastatin (Sigma, St. Louis, MO, USA). Animals were killed with pentobarbital 14 days after the operation. Successful ureteral ligation was confirmed at death. Both kidneys from obstructed animals and the left kidney of the control group were decapsulated, removed, and weighed.

Identification of cellular apoptosis: TUNEL technique

After the digesting and quenching steps, equilibration buffer was applied directly to tissue sections and working strength TdT enzyme was then applied directly. A biotin-conjugated anti-digoxigenin antibody (Sigma) was used. Afterwards tissue sections were incubated with biotinylated anti-mouse IgG (Dako, Carpinteria, CA, USA) at 1:100 dilution for 45 min at room temperature and later with peroxidase-labeled streptavidin (strept AB Complex/HRP, Dako) at 1:100 dilution for 45 min at room temperature. After a brief wash, 3.3'-diaminobenzidine tetrahydrochloride (0.5

TABLE 1.

Primers designed from rat sequences for RT-PCR

Primer	Sequence	Annealing	Predicted Product Size, (bp)
WT-1 Antisense Sense	5′-GACCTGAACGCGCTGCTG-3′ 5′-CTGCTCCTCGTGCGGCTC-3′	52ºC	240/246*
p53 Antisense Sense	5′-TCTGTCATCTTCCGTCCCTTC TC-3′ 5′-AACACGAACCTCAAAGCTGCTGTCCCG-3′	52ºC	547
iNOS Antisense Sense	5′-GCATGGACCAGTATAAGGCAAGCA-3′ 5′-GCTTCTGGTCGATGTCATGAGCAA-3′	55ºC	222
Hsp70 Antisense Sense	5′-CCGCCTACTTCAACGACTC-3′ 5′-TCTTGAACTCCTCCACGAAG-3′	56ºC	291
BcL2 Antisense Sense	5′-CTTGTGGCCCAGGTATGC-3′ 5′-ATGGCGCAAGCCGGGAGAA-3	59ºC	708
Bax Antisense Sense	5′-TCAGCCCATCTTCTTCCAGAT-3′ 5′-ATGGACGGGTCCGGGGAGC-3′	59ºC	550
β-Actin Antisense Sense	5′-GTGCCACCAGACAGCACTGTGTTG-3′ 5′-TGGAGAAGAGCTATGAGCTGCCTG-3′	65ºC	201

* 246 bp are predicted product size in canine (MDCK cell)

mg/ml)/H_2O_2 (0.01%) was added. Sections from involuting prostates (n = 2) were used as a positive control. For quantification of apoptotic epithelial cells in cross sectioned cortex areas, ten consecutive fields were randomly selected and evaluated at 400X, on a 10 x 10 grid using an image analyzer (Scion Image for Windows, Scion Corporation USA).

RT-PCR for WT-1, p53, iNOS, Hsp70, BcL_2 and Bax

Total RNA was obtained by using Trizol reagent (Gibco BRL). One microgram of total RNA was denatured in the presence of 0.5 µg/50µl Oligo $(dT)_{15}$ primer and 40 U recombinant ribonuclease inhibitor RNasin (Promega, USA). Reverse transcription was performed using 200 units of reverse transcriptase M-MLV RT in reaction buffer, 0.5 mM dNTPs each, and incubated for 60 min at 42ºC. The cDNA (10 µl) was amplified by polymerase chain reaction (PCR) in standard conditions. Each sample was processed for WT-1, p53, iNOS, Hsp70, BcL_2, Bax and β-actin with primers indicated in Table 1. Specific signals were standardized against β-actin signal for each sample, and results were expressed as a ratio (relative densitometry units or RDU). To ensure that PCR was performed in the linear amplification range, samples were taken after 20, 25, 30, 35 and 40 cycles. The reaction was linear over this range for WT-1 (r=0.93), p53 (r=0.90), iNOS (r=0.91), Hsp70 (r=0.92), BcL_2 (r=0.89), Bax (r=0.90) and β-actin (r=0.85).

Determination of nitrite levels

Nitrite levels were measured in renal cortex and epithelial cell lysates according to Griess (1864), with minor modifications. Homogenates from renal cortex tissue and epithelial cells were centrifuged at 6,400 rpm for 20 minutes; 100 µl supernatants were used for the NO production assay. The amount of NO_2^- was adjusted according to the protein amount by the Bradford method. Nitrite was measured by a spectrophotometer Shimadzu UV-160 (Shimadzu Corporation, Kyoto, Japan) at a wavelength of 540 nm. The NO_2^- present was expressed as nmol of nitrite generated per µg protein per 100 µl homogenate.

Kidney epithelial cell culture

Madin-Darby canine kidney (MDCK) cells were cultured in Dulbecco´s Modified Eagle Medium (Gibco™) to which 10% fetal bovine serum (FBS) was added. The medium was supplemented with bicarbonate, glutamine and penicillin-streptomycin (50 µg/ml). Following passage, the number of viable cells was determined by the trypan blue exclusion method. Cell counts were calculated on the average of six separate readings and expressed in millions per ml (10^6 cells/ml). After 80% confluence, cells were placed in quiescence (0.1% FBS) for 24 hours. Immediately, the pharmacological protocol was started. NG-nitro-L-arginine methyl ester (1 mmol/l), L-arginine (1 mmol/l) and sodium nitroprusside (0.15 and 0.25 mmol/l) were added to the culture medium for 72 hours. The culture medium containing the drugs was replaced every 24 hours. All treatments were done in triplicate, and all experiments were replicated three to five times. The experimental time was set at 72 hours because MDCK cells showed a log phase of 2 days during which cell concentration decreased to 100,000 cells/ml. After this log phase, cells entered the exponential growth phase with a mean doubling time of 12 hours. Cells were collected for total RNA isolation and nitric oxide analysis by Griess reaction.

TABLE 2.

Kidney mass/body mass in obstructed and sham-operated rats (5 per group). Values are mean ± SEM (n = 5).

Control (mg/g)	Control + rosuvastatin (mg/g)	Obstructed (mg/g)	Obstructed + rosuvastatin (mg/g)
6.50 ± 0.20	6.15 ± 0.18	5.60 ± 0.15**	5.80 ± 0.22*

** obstructed rats vs. control, p<0.01, 14 days after operation.

* rosuvastatin-treated obstructed rats vs. non-treated obstructed rats, p<0.05, 14 days after operation.

"In vivo" administration of L-arginine and NG-nitro-L-arginine methyl ester in neonatal unilateral ureteral obstruction

In order to test whether WT-1 mRNA expression is modulated by nitric oxide levels associated to stimulation of Hsp70, NG-nitro-L-arginine methyl ester (50 mg/kg/day) (Kawaguchi *et al.,* 1999) or L-arginine (100 mg/kg/day) (Schlaich *et al.,* 2007) was administrated by gavage for 14 days to neonatal rats previously subjected to sham operation or complete unilateral ureteral obstruction within the first 48 hours of life.

Statistical analysis

Results are given as mean ± standard error of mean (SEM). Comparisons between groups were assessed by one-way analysis of variance followed by Bonferroni's test. A $p<0.05$ was considered to be significant. GraphPad InStat version 3.00 for Windows 95 (Graph Pad Software, Inc, San Diego, CA, USA) was used.

Results

Kidney mass / body mass ratio

As shown in Table 2, there was a significant decrease in kidney mass/body mass ratio in the obstructed vs. the control group (5.60 ± 0.15 vs. 6.50 ± 0.20). Rosuvastatin treatment for 14 days attenuated this effect (5.80 ± 0.22 vs. 5.60 ± 0.15, treated obstructed kidneys compared with non-treated obstructed kidneys, respectively). In non-obstructed rats, no difference in kidney mass/body mass ratio was observed following 14 days of rosuvastatin treatment when compared to the control group (6.15 ± 0.18 vs. 6.50 ± 0.20).

Rosuvastatin effect in mitochondrial apoptosis modulation during neonatal unilateral ureteral obstruction

Figure 1 shows that mRNA Bax/BcL_2 ratio was significantly raised in renal cortex from neonatal unilateral ureteral obstruction for 14 days related to control (1.20 ± 0.03 vs. 0.80 ± 0.05). Conversely, in rats with unilateral ureteral obstruction, cortex fractions from rosuvastatin-treated animals showed a decreased mRNA Bax/BcL_2 ratio compared with vehicle-treated animals (0.60 ± 0.10 vs. 1.20 ± 0.03). Also, figure 2 shows in parallel histological studies an increased number of confirmed TUNEL-positive apoptotic cells and stronger apoptosis molecular markers in tubular epithelial cells from cortex of vehicle-treated obstructed animals compared to control (18 ± 6 vs. 3.50 ± 0.15). In obstructed rats, TUNEL-positive apoptotic cells were significantly less in rosuvastatin-treated animals compared with vehicle-treated animals (7 ± 2 vs. 18 ± 6).

WT-1 and p53 RT-PCR expression in neonatal unilateral ureteral obstruction

Figure 3 shows a large decline in WT-1 mRNA expression in renal cortex from neonatal unilateral ureteral obstruction for 14 days compared to control (0.50 ± 0.05 vs. 0.90 ± 0.05), as well as a significant reduction in p53 expression (0.55 ± 0.04 vs. 0.80 ± 0.04). Rosuvastatin treatment significantly reversed the decrease in WT-1 and p53 mRNA expression associated with obstruction (0.85 ± 0.06 vs. 0.50 ± 0.05 and 0.95 ± 0.05 vs. 0.55 ± 0.04, respectively). No difference was observed in WT-1 mRNA expression between rosuvastatin-treated control and untreated control animals (0.92 ± 0.04 vs. 0.90 ± 0.05) but p53 was increased in the former (1.20 ± 0.06 vs. 0.80 ± 0.04).

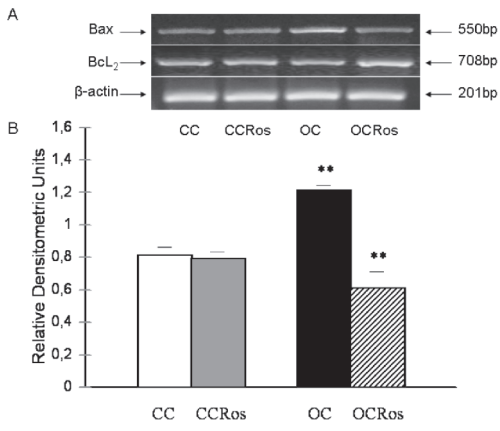

FIGURE 1. *Mitochondrial apoptotic pathway induction in rosuvastatin-treated rats with obstructed kidneys. Bax/BcL_2 RT-PCR expression.* Induction of mRNA expression for BcL_2 and Bax and the ratio of mRNA Bax / mRNA BcL_2 in obstructed kidney cortexes with and without rosuvastatin treatment. Top panel: Representative gels of BcL_2 and Bax mRNA. The corresponding housekeeping β-actin is included below (A). Bottom panel: Histograms show the relative concentration of mRNAs for BcL_2 and Bax to β-actin mRNA. Increased Bax / BcL_2 ratio in OC vs. CC, ** $p<0.01$. Rosuvastatin decreased Bax / BcL_2 ratio in OCRos vs. OC, ** $p<0.01$ (B). Data represent the mean ± SEM of five independent experiments.

Nitric oxide bioavailability is associated with rosuvastatin treatment during neonatal unilateral ureteral obstruction

As shown in Figure 4, there is a low iNOS mRNA expression in obstructed cortex compared with control cortex (0.30 ± 0.03 vs. 0.75 ± 0.05). Meanwhile, in obstructed animals, iNOS mRNA expression in the cortex was significantly higher in rosuvastatin-treated rats (0.83 ± 0.06 vs. 0.30 ± 0.03). In addition, decreased nitrite levels were shown in obstructed cortex related to control cortex (40 ± 7 vs. 80 ± 7 nmol NO_2 generated / µg protein / 100 µl homogenate). Pre-treatment with rosuvastatin enhanced nitrite levels in obstructed cortex versus vehicle treated obstructed rats (78 ± 6 vs. 40 ± 7 nmol NO_2 generated / µg protein / 100 µl homogenate).

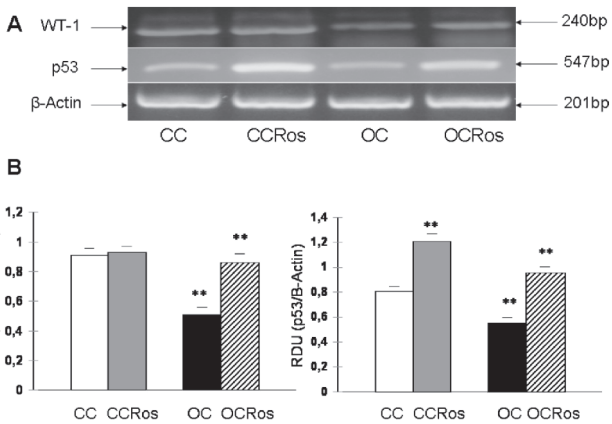

FIGURE 3. *WT-1 and p53 RT-PCR expression in neonatal unilateral ureteral obstruction. Rosuvastatin effect*
A-Representative gels of WT-1 (240bp) and p53 (547bp) mRNA in cortex from obstructed and sham rats pretreated with rosuvastatin; are shown. The corresponding housekeeping β-actin is included below.
B-Graphical representation of WT-1/β-actin mRNA ratio and p53/β-actin mRNA ratio showed lower mRNA expression in 14 day obstructed cortex (OC) vs control cortex (CC), (**p<0.01, in both). Conversely, after rosuvastatin treatment higher WT-1 mRNA expression and p53 mRNA expression were shown in OCRos vs OC, (**p<0.01, in both). In addition, p53 mRNA was inducted in cortex from sham rats pretreated with rosuvastatin (CCRos vs CC, **p<0.01). Results are means ± SEM of five independent observations.

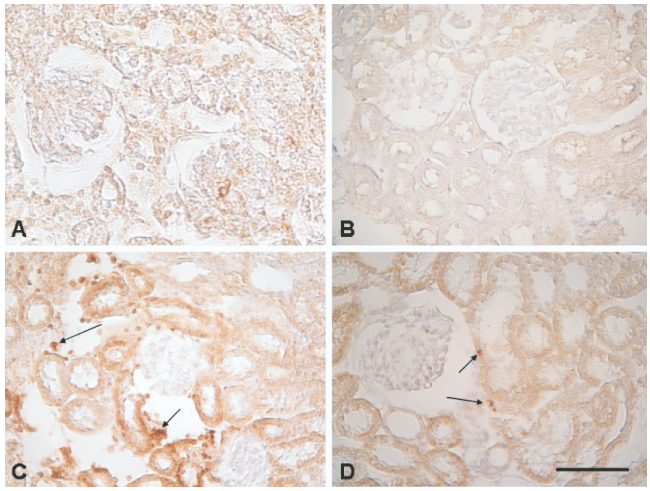

FIGURE 2. *Histologic sections of neonatal cortex kidney following unilateral obstruction for 14 days. Rosuvastatin cytoprotective effect.*Localization of apoptotic nuclei by TdT-uridine-nick-end-labeling technique: apoptotic nuclei appear as heavy brown-staining nuclei in tubule epithelial cells (arrows).
Fourteen day kidney cortex from non-treated (A) and rosuvastatin-treated sham rats (B). Apoptotic cells are rarely seen in tubule epithelial cells. Fourteen days after ipsilateral obstruction in cortex kidney, apoptotic nuclei appear as heavy brown-staining nuclei in dilated cortical collecting ducts and in lesser proportion in proximal tubules (C). Slight increase of apoptotic cells in epithelium from dilated cortical collecting ducts and proximal tubules, in kidney cortex from rosuvastain-treated UUO rats (D). Bar = 100 µm in A, B, C and D.

Rosuvastatin and heat shock response during neonatal unilateral ureteral obstruction

In figure 5, a lower Hsp70 expression can be seen in obstructed cortex from non-treated rats compared to control rats (0.35 ± 0.05 vs. 0.95 ± 0.05). In contrast, an increase in Hsp70 expression was found when comparing cortex from obstructed rats between those pretreated with rosuvastatin vs. untreated ones (0.96 ± 0.11 vs. 0.35 ± 0.05). No difference was found between rosuvastatin-treated control and untreated control (0.96 ± 0.06 vs. 0.95 ± 0.05).

Nitric oxide bioavailability modulates Hsp70 and WT-1 mRNA expression in MDCK cells

As shown in figure 6, nitric oxide level measured as nitrite was markedly low when MDCK cells were treated with NG-nitro-L-arginine methyl ester (nitric oxide inhibitor) for 72 hours (15 ± 5 vs. 150 ± 20 nmol NO_2 generated / µg protein / 100 µl homogenate). Con-

versely, L-arginine (nitric oxide-inducing amino acid), increased nitrite level over that of control MDCK cells (200 ± 15 vs. 150 ± 20 nmol NO_2 generated / µg protein / 100 µl homogenate). The nitric oxide donor sodium nitroprusside very significantly increased nitrite levels in MDCK cells (230 ± 20 vs. 150 ± 20 nmol NO_2 generated / µg protein / 100 µl homogenate) when present at a concentration of 0.25 mmol/L. However, it had no significant effect at 0.15 mmol/L (170 ± 15 vs. 150 ± 20 nmol NO_2 generated / µg protein / 100 µl homogenate).

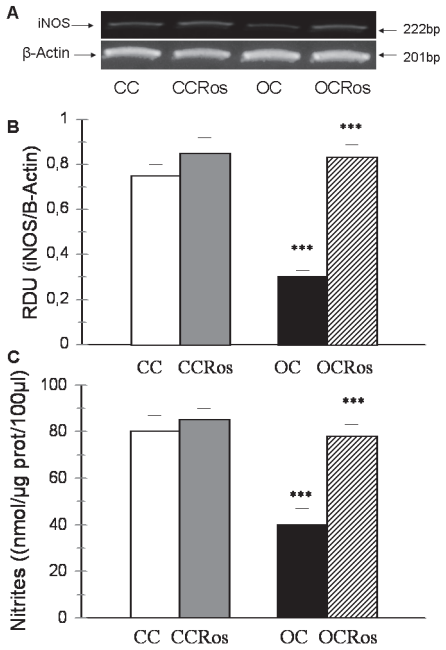

FIGURE 4. *Nitric oxide bioavailability is associated with rosuvastatin treatment during neonatal unilateral ureteral obstruction.* Endogenous nitric oxide generation and iNOS expression in kidney cortex after 14 days of unilateral ureteral obstruction. A- Representative gel of iNOS mRNA in control and obstructed kidney cortexes after rosuvastatin treatment. Housekeeping gene β-actin expression is shown in the line below, in the same order as the densitometry bars. B-Graphical representation of iNOS/β-actin mRNA ratio showed a decreased expression of iNOS isoform in obstructed cortex (OC) vs control cortex (CC) (***p<0.001) after 14 days of obstruction. Contrarily, increased iNOS expression from rosuvastatin-treated obstructed rats (OCRos) compared to obstructed cortex (OC); (***p<0.001), was shown.
C- Measurement of nitrite generated (nmol NO_2 generated/mg protein/100 µl homogenate). Following 14 days of obstruction decreased nitrites in OC vs. CC (***p<0.001). Obstructed cortex from neonatal rats pretreated with rosuvastatin resulted in higher nitrite levels related to obstructed non-treated rats (***p<0.001).
Results are means ± SEM of five independent observations.

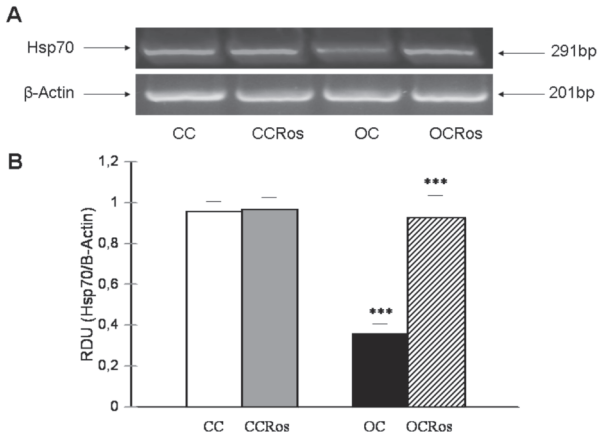

FIGURE 5. *Heat shock response in rosuvastatin-treated neonatal unilateral ureteral obstruction. mRNA expression of Hsp70.* A-Representative gel of Hsp70 (291bp) mRNA in cortex from obstructed and sham rats pretreated with rosuvastatin are shown. The corresponding housekeeping β-actin is included below. B-Graphical representation of Hsp70/β-actin mRNA ratio showed lower mRNA expression in 14 day obstructed cortex (OC) vs control cortex (CC) (***p<0.001). Conversely, after rosuvastatin treatment higher Hsp70 mRNA expression were shown in OCRos vs OC (***p<0.001). Results are means ± SEM of five independent observations.

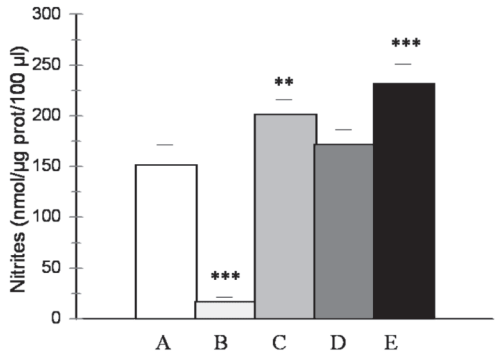

FIGURE 6. *Nitric oxide bioavailability in MDCK cells.* A-The bar represents nitrite levels in 72 hours of control MDCK cells. B-The bar represents nitrite levels in 72 hours of NG-nitro-L-arginine methyl ester treated MDCK cells. Nitrite measurements were diminished when MDCK cells were NG-nitro-L-arginine methyl ester treated versus control MDCK cells (*** p<0.001). C-The bar represents nitrite levels in 72 hours of L-arginine treated MDCK cells. L-arginine reversed nitrite levels on top of the control MDCK cells (**p<0.01).
D-The bar represents nitrite levels in 72 hours of sodium nitroprusside (0.15 mmol/L) treated MDCK cells. No difference in sodium nitroprusside 0.15 mmol/L treatment MDCK cells with respect to control MDCK cells p=ns was shown. E-The bar represents nitrite levels in 72 hours of sodium nitroprusside (0.25 mmol/L) treated MDCK cells. The nitric oxide donor at 0.25 mmol/L significantly increased the nitrite levels in MDCK cells with respect to controls non-treated (***p<0.001). Results (A, B, C, D, and E) are means ± SEM of three independent observations.

On the other hand, figure 7 shows that Hsp70 mRNA expression was significantly decreased when MDCK cells were treated with NG-nitro-L-arginine methyl ester (0.32 ± 0.05 vs. 0.85 ± 0.03). L-arginine treatment did not alter Hsp70 expression (0.83 ± 0.04 vs. 0.85 ± 0.03). Likewise, no significant differences in Hsp70 expression was found when cells were treated for 72 hours with sodium nitroprussideat any concentration (0.75 ± 0.05 vs. 0.85 ± 0.03 for 0.15 mmol/L and 0.79 ± 0.02 vs. 0.85 ± 0.03 for 0.25 mmol/L, respectively).

Figure 8 shows that WT-1 mRNA expression was decreased in MDCK cells treated with NG-nitro-L-arginine methyl ester (0.40 ± 0.04 vs. 0.80 ± 0.05), while L-arginine had no significant effect (0.90 ± 0.07 vs. 0.80 ± 0.05). Nevertheless, sodium nitroprusside treatment at both concentrations promoted an increase in WT-1 mRNA expression in MDCK cells (1.05 ± 0.07 vs. 0.80 ± 0.05 for 0.15 mmol/L and 0.98 ± 0.06 vs. 0.80 ± 0.05 for 0.25 mmol/L).

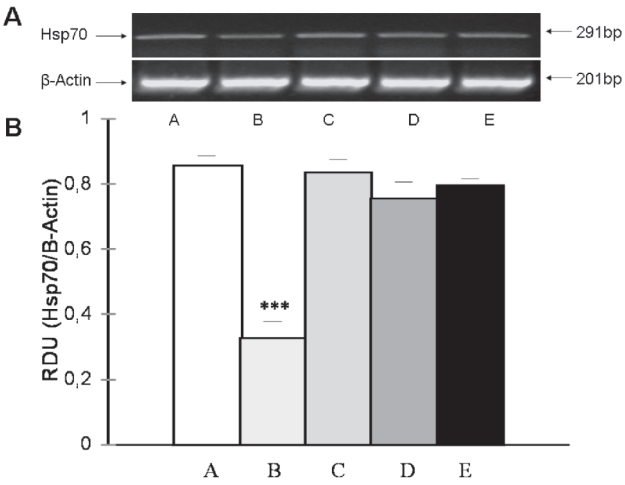

FIGURE 7. *Nitric oxide bioavailability modulates Hsp70 expression in MDCK cells.* A-Representative blot of Hsp70 (291bp) mRNA in control (A), NG-nitro-L-arginine methyl ester (B), L-arginine (C), sodium nitroprusside 0.15 mmol/L (D) and sodium nitroprusside 0.25 mmol/L (E) MDCK cells treated, are shown. The corresponding housekeeping β-actin is included below. B-Graphical representation of Hsp70/β-actin mRNA ratio. Hsp70 mRNA expression was significantly decreased when MDCK cells were treated with NG-nitro-L-arginine methyl ester with respect to non-treated MDCK cells (***p<0.001) (B). Whereas L-arginine treatment did not alter the Hsp70 expression compared to untreated MDCK cells, p=ns (C). No differences were observed in Hsp70 expression when cells were treated with sodium nitroprusside at any dose compared to the non-treated MDCK cells, p=ns (D and E). Results (A, B, C, D, and E) are means ± SEM of three independent observations.

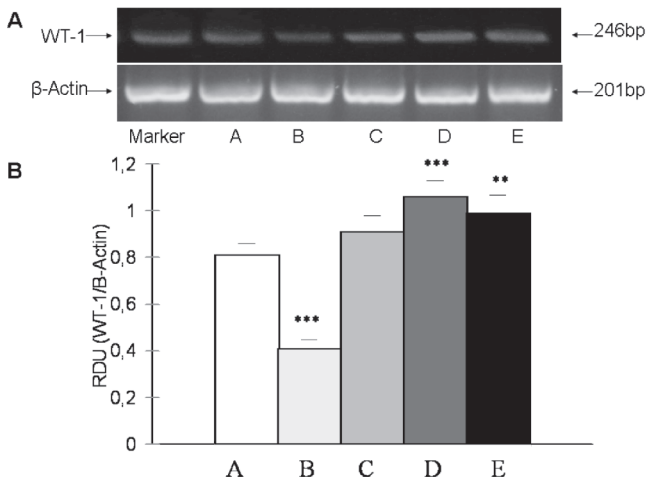

FIGURE 8. *Nitric oxide bioavailability modulates WT-1 expression in MDCK cells.* A-Representative blot of WT-1 (246bp) mRNA in control (A), NG-nitro-L-arginine methyl ester (B), L-arginine (C), sodium nitroprusside 0.15 mmol/L (D) and sodium nitroprusside 0.25 mmol/L (E) MDCK cells treated, are shown. The corresponding housekeeping β-actin is included below. B-Graphical representation of WT-1/β-actin mRNA ratio. WT-1 mRNA expression was significantly decreased when MDCK cells were treated with NG-nitro-L-arginine methyl ester with respect to non-treated MDCK cells (***p<0.001) (B). No difference in L-arginine treated MDCK cells with respect to control MDCK cells, p=ns (C), was shown. Sodium nitroprusside (0.15 mmol/L and 0.25 mmol/L) promoted a significant increased in WT-1 expression in MDCK cells compared to untreated MDCK cells (***p<0.001 and **p<0.01 (D and E), respectively). Results (A, B, C, D, and E) are means ± SEM of three independent observations.

In vivo administration of L-Arginine and NG-nitro-L-arginine methyl ester in unilateral ureteral obstruction. Effects on WT-1 mRNA expression in obstructed cortex from neonatal rats

In vivo administration of L-Arginine for 14 days resulted in higher nitrite levels (95 ± 6 vs 75 ± 4 nmol NO_2 generated / µg protein / 100 µl homogenate), increased WT-1 mRNA expression (1.22 ± 0.06 vs 0.90 ± 0.05) and higher Hsp70 mRNA expression levels (1.1 ± 0.06 vs 0.95 ± 0.04) in control cortex homogenates (Fig. 9). Since data showed that L-Arginine induced Hsp70 / WT-1 expression, whether pre-treatment with L-Arginine in the obstructed cortex homogenate resulted in WT-1 and Hsp70 mRNA modulation was next assessed.

Obstructed kidney homogenates showed higher nitrite levels (73 ± 5 vs 45 ± 5 nmol NO_2 generated / µg protein / 100 µl homogenate), increased WT-1 mRNA expression (0.92 ± 0.06 vs 0.61 ± 0.04), as well as higher Hsp70 mRNA expression (1.00 ± 0.06 vs 0.65 ± 0.04) in L-Arginine pretreated animals.

Conversely, treatment of control rats with NG-nitro-L-arginine methyl ester was associated with lower nitrite levels (48 ± 7 vs 75 ± 4 nmol NO_2 generated / µg protein / 100 µl homogenate), decreased WT-1 mRNA expression (0.43 ± 0.03 vs 0.9 ± 0.05) and lower Hsp70 mRNA expression (0.75 ± 0.05 vs 0.95 ± 0.04). Obstructed kidney homogenates from NG-nitro-L-arginine methyl ester-pretreated rats showed lower nitrite levels (35 ± 4 vs 45 ± 5 nmol NO_2 generated / µg protein / 100 µl homogenate), WT-1 mRNA expression (0.40 ± 0.02 vs 0.61 ± 0.04) and Hsp70 mRNA expression (0.35 ± 0.03 vs 0.65 ± 0.04).

Discussion

Changes in gene expression patterns during development and maturation of the kidney modulate a series of events that are responsible for extraordinary structural and functional complexity. Congenital obstructive nephropathy disrupts normal renal development and causes chronic progressive interstitial fibrosis, which contributes to renal growth arrest, ultimately leading to chronic renal failure. Therefore, renal growth and development are severely affected by obstructive injury through complex interactions among regulators of cell proliferation and apoptosis. A large number of cell types present in the early embryonic kidney are not found at later stages of development, with apoptosis as the main mechanism for cell selection. Normal kidney development requires the conversion of mesenchymal cells into polarized epithelial cells (Ekblom, 1989), an exceptional process during nephrogenesis. Many genes play relevant functions in kidney development. The *wt-1* gene is expressed in a large variety of embryonic tissues, suggesting that it is involved in a wide spectrum of developmental events. In addition, *wt-1* appears to be the key determinant of the startup sequence for mesenchymal-epithelial conversion, and it was identified as missing or mutated in embryonic kidney cancer cells (Buckler *et al*., 1991). WT-1 is a Cys-His zinc finger polypeptide which appears to be a transcription factor controlling gene expression during embryonic kidney development. One of the more significant results in the field of renal development was the finding that knocking out the *wt-1* gene in mice results in anephric animals (Kreidberg *et al*., 1993).

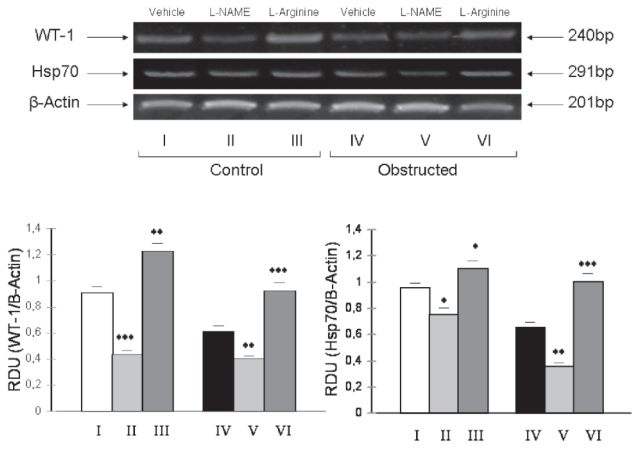

FIGURE 9. *In vivo administration of L-Arginine and NG-nitro-L-arginine methyl ester in neonatal unilateral ureteral obstruction.* A-Representative gels of WT-1 (240bp) and Hsp70 (291bp) mRNA in cortex from obstructed and sham operated rats pretreated or not with L-Arginine and NG-nitro-L-arginine methyl ester, are shown. The corresponding housekeeping β-actin is included below. B- In NG-nitro-L-arginine methyl ester pre-treatment during 14 days, graphical representation of WT-1/β-actin mRNA ratio and Hsp70/β-actin mRNA ratio showed lower expression in obstructed cortex as well as in control cortex. Conversely, after L-arginine pre-treatment, higher WT-1 mRNA expression and Hsp70 mRNA expression were shown in obstructed cortex as well as in control cortex. Results are means ± SEM of five independent observations.

During congenital obstructive nephropathy, major regulators of mesenchymal-epithelial transformation and renal tubular development, such as *wt-1* and *sall1*, are decreased (Liapis, 2003). However, the exact mechanisms of these decreases are not yet known; much less which additional factors might be implicated in the natural course of congenital obstructive nephropathy. In this setting, we propose that WT-1 mRNA expression may be involved. The present work was designed to evaluate and discuss these topics of interest, highlighting that WT-1 mRNA expression might be modulated by nitric oxide availability and Hsp70 interaction in the context of neonatal unilateral ureteral obstruction.

It has been hypothesized that nitric oxide may participate in the maintenance of renal function and in the maturation of developing kidneys (Elli *et al*., 2005; Forbes *et al*., 2007). Nitric oxide bioavailability has been associated to renoprotection by statins (Zhou *et al*., 2008). Moreover, rosuvastatin (hydrophilic statin) may have potential utility as a therapeutic option in renal diseases that are characterized by inflammation and fibrosis, independently of changes in blood pressure and plasma lipid levels (Gianella *et al*., 2007). Randomized studies with HMG-CoA reductase inhibitors (statins) have shown that their major adverse effects are associated with muscle and liver toxicity. However, rosuvastatin

seems safe in this regard (Shepherd *et al*., 2004). In addition, we found no significant changes in either body mass or kidney mass in rosuvastatin-treated animals.

Apoptosis is the principal mechanism that leads to tubular atrophy during the neonatal obstructive nephropathy process. Excessive cell death is mediated by decreased expression of apoptosis inhibiting gene like BcL_2 and over expression of pro-apoptotic genes like Bax. Correspondingly, 14 days of obstruction led to the induction of apoptosis regulated by mitochondrial signal pathway through a pro-apoptotic increased Bax/BcL_2 ratio and, consequently, an increased activity of caspase 3 (Manucha and Vallés, 2008). Results agree with the decreased WT-1 expression previously reported by other authors and confirmed by us. Therefore, it may be suggested that in certain cellular contexts, WT-1 exhibits antiapoptotic potential through the transcriptional upregulation of BcL_2 (Mayo *et al*., 1999).

In the present study, apoptosis induction in neonatal obstructed cortex was associated with significantly low WT-1 mRNA expression and nitric oxide bioavailability, as shown by poor iNOS expression and low nitrite levels. Accordingly, a substantial decrease in kidney mass / body mass ratio was found in obstructed neonatal rats. Rosuvastatin treatment did not change this ratio. Future experiments may explore WT-1 mRNA expression during unilateral ureteral obstruction after release and its possible involvement in renal functional and structural recovery.

Recently, Yoo *et al*. (2010) reported that, in complete unilateral ureteral obstruction in mice, iNOS attenuates apoptosis and increases renal parenchymal thickness. Other results corroborate previous reports in which nitric oxide has been proposed as a key factor modulating apoptosis in unilateral ureteral obstruction (Miyajima *et al*., 2001; Ito *et al*., 2005; Manucha, 2007; Manucha and Vallés, 2008). Moreover, a functional WT-1/iNOS promoter interaction where iNOS promoter is strain-dependently regulated, which may relate to strain-dependent differences in WT-1 transcription factor expression (Johannesen *et al*., 2003).

Many studies have shown the benefits of nitric oxide donors and deleterious effects of nitric oxide inhibitors in obstructive nephropathy. Nevertheless, to our knowledge this is the first study that attempts to establish the involvement of nitric oxide modulation of WT-1 mRNA expression during neonatal unilateral ureteral obstruction. Thus, with rosuvastatin treatment, a significant decrease of apoptosis in obstructed cortex was found, linked to increased nitric oxide availability and associated with increased WT-1 mRNA expression. These results suggest that treatment with rosuvastatin might slow or even reverse the process of apoptosis during neonatal unilateral ureteral obstruction.

Nitric oxide stimulates the expression of enzymes and transcription factors involved in DNA repair and modulation of apoptosis, such as the tumor suppressor p53. In turn, p53 interacts with WT-1 and modulates its ability to regulate the transcription of its respective target genes (Scharnhorst *et al*., 2000). Consequently, it might be expected that increased nitric oxide availability induces p53 and WT-1 mRNA expression, as indeed shown by our results. Moreover, WT-1 can stabilize p53, adjust its trans-activational properties, and inhibit its ability to induce apoptosis without affecting cellular arrest (Maheswaran *et al*., 1995). This effect may explain the elevated p53 levels observed by other authors during obstructive nephropathy apoptosis induction (Cummings, 1996; Morrissey and Klahr, 1999; Miyajima *et al*., 2000; Topcu *et al*., 2008). Furthermore, nitric oxide treatment preserves vascular smooth muscle cells from mitochondrial-dependent apoptosis and drives cells to quiescence through an increase in p53 (Duran *et al*., 2009). Therefore, it appears that nitric oxide/p53/WT-1 interactions can modulate the WT-1 expression and/or function.

Nitric oxide can oxidize intracellular glutathione and modify cellular antioxidant levels. This stimulates heat shock protein induction as in Hsp32 and Hsp70. Nitric oxide generated from several compounds induces Hsp70 expression in arterial smooth muscle cells (Xu *et al*., 1997). Hsp70 plays an important role in nascent protein folding, reassembling denatured proteins and solubilized protein aggregates (Goloubinoff and De Los Rios, 2007). Moreover, it is involved in cellular signaling pathways linked to apoptosis, membrane translocation and in the modulation of the activities of tumor suppressor proteins, including p53 and WT-1 (Cheng *et al*., 2001). Inducible Hsp70 expression has been shown to enhance the survival of mammalian cells exposed to numerous types of stimuli that induce stress and apoptosis (Jäättelä, 1999). Furthermore, inducible Hsp70 protects renal epithelial cells from apoptosis by caspase activation (Li *et al*., 2002).

WT-1 and Hsp70 are physically associated in embryonic rat kidney cells, where the amino-terminal transactivation domain of WT-1 is required for binding to Hsp70, and domain expression itself is sufficient to induce Hsp70 expression (Maheswaran *et al*., 1998). Present work shows a significant decrease in Hsp70 expression associated with low nitric oxide availability in the neonatal obstructed cortex. Interestingly, rosuvastatin treatment induced a significant increase in

iNOS expression and nitrite levels, and this was related to enhance Hsp70 expression.

Induction of Hsp70 not only protects cells from damage due to apoptosis induction, but also from damage due to oxidative injury. Hence, nitric oxide can induce cytoprotection in early obstructed kidney cortex tubular epithelial cells through the stimulation of Hsp70 expression (Manucha and Vallés, 2008).

Previously, Hegarty *et al.* (2002) showed in a well-characterized renal epithelial cell line (MDCK) under mechanical strain (hydronephrosis cellular model) an increased susceptibility to apoptosis. The cellular effects of mechanical strain were reversed by sodium nitroprusside and L-arginine.

Since about 80% of total kidney mass is composed of tubular epithelial cells, it seems reasonable to infer that *in vivo* results represent mostly phenomena affecting this cell population. To corroborate this, basal WT-1 mRNA expression and the effects of nitric oxide availability were also studied *in vitro* in MDCK cells. Here, low nitric oxide availability was associated with low expression of Hsp70 and WT-1. Nitric oxide donors did not significantly change the Hsp70 expression, nor did the nitric oxide precursor L-arginine induce any changes in WT-1 mRNA expression. However, WT-1 mRNA expression was increased when MDCK cells were incubated for 72 hours with nitric oxide donors. These results in MDCK cells suggest a greater susceptibility to low nitric oxide levels associated with low WT-1 mRNA expression. Further work should be done to establish these differences.

While it cannot be simply assumed that *in vitro* results faithfully reproduce *in vivo* mechanisms, in both cases nitric oxide levels associated with Hsp70 mRNA expression seems to modulate WT-1 mRNA expression. This proposed mechanism is further supported by the results of treating neonatal rats with nitric oxide modulators, where a close relationship between endogenous nitrite levels and WT-1/Hsp70 mRNA expression was found.

The p53 protein interacts with members of the Hsp70 chaperone family which can regulate its function (Lane *et al*., 1993; Takenaka *et al*., 1995). In this regard, neonatal unilateral ureteral obstruction shows low p53 and Hsp70 expressions, which are increased in association with higher nitric oxide levels under rosuvastatin treatment. Conversely, MDCK cells with nitric oxide deprivation expressed low Hsp70 and p53 mRNA levels. These observations suggest a potential role for nitric oxide bioavailability and Hsp70 interaction during kidney differentiation.

Collectively, our present findings suggest that rosuvastatin may modulate WT-1 mRNA expression through renal nitric oxide bioavailability associated with Hsp70 interaction, preventing obstruction-induced cell death during neonatal unilateral ureteral obstruction.

Acknowledgements

The authors gratefully acknowledge comments and contributions of Dr. Fernando Saraví. This work was performed with grant from the Research and Technology Council of Cuyo University (SeCyT) Mendoza, Argentina/N: 882/07 and Florencio Fiorini Foundation awarded to Walter Manucha.

References

Buckler AJ, Pelletier J, Haber DA, Glaser T, Housman DE (1991). Isolation, characterization, and expression of the murine Wilms' tumor gene (WT-1) during kidney development. *Molecular and Cellular Biology* **11**: 1707-1712.

Chan W, Krieg RJ Jr, Ward K, Santos F Jr, Lin KC, Chan JC (2001). Progression after release of obstructive nephropathy. *Pediatric Nephrology* **16**: 238-44.

Cheng H, Cenciarelli C, Shao Z, Vidal M, Parks WP, Pagano M, Cheng-Mayer C (2001). Human T cell leucemia virus type 1 Tax associates with a molecular chaperone complex containing hTid-1 and Hsp70. *Current Biology* **11**: 1771-1775.

Chevalier RL (1998). Pathophysiology of obstructive nephropathy in the newborn. *Seminars in nephrology* **18**: 585-593.

Chevalier RL, Thornhill BA, Chang AY, Cachat F, Lackey A (2002). Recovery from release of ureteral obstruction in the rat: relationship to nephrogenesis. *Kidney International* **61**: 2033-2043.

Cormack-Aboud FC, Brinkkoetter PT, Pippin JW, Shankland SJ, Durvasula RV (2009). Rosuvastatin protects against podocyte apoptosis *in vitro*. *Nephrology Dialysis and Transplantation* **24**: 404-412.

Cummings MC (1996). Increased p53 mRNA expression in liver and kidney apoptosis. *Biochimica et biophysica acta* **1315**: 100-104.

Duran X, Vilahur G, Badimon L (2009). Exogenous *in vivo* NO-donor treatment preserves p53 levels and protects vascular cells from apoptosis. *Atherosclerosis* **205**: 101-106.

Ekblom P (1989). Developmentally regulated conversion of mesenchime to epithelium. *The FASEB journal* **3**: 2141-2160.

Elli M, Söylemezoglu O, Erbas D, Bakkaloglu SA, Buyan N, Ozkaya O, Hasanoglu E (2005). Plasma and urine nitric oxide levels in healthy Turkish children. *Pediatric Nephrology* **20**: 1605-1609.

Forbes MS, Thornhill BA, Park MH, Chevalier RL (2007). Lack of endothelial nitric-oxide synthase leads to progressive focal renal injury. *American Journal of Pathology* **170**: 87-99.

Gianella A, Nobili E, Abbate M, Zoja C, Gelosa P, Mussoni L, Bellosta S, Canavesi M, Rottoli D, Guerrini U, Brioschi M, Banfi C, Tremoli E, Remuzzi G, Sironi L (2007). Rosuvastatin treatment prevents progressive kidney inflammation and fibrosis in stroke-prone rats. *American Journal of Pathology* **170**: 1165-1177.

Goloubinoff P, De Los Rios P (2007). The mechanism of Hsp70 chaperones: (entropic) pulling the models together. *Trends in biochemical sciences* **32**: 372-380.

Griess JO (1864). On a new series of in which nitrogen is substituted for hydrogen. *Philosophical transactions of the Royal Society of London. Series B, Biological sciences* **154**: 667-731.

Hegarty NJ, Watson RW, Young LS, O'Neill AJ, Brady HR, Fitzpatrick JM (2002). Cytoprotective effects of nitrates in a cellular model of hydronephrosis. *Kidney International* **62**: 70-77.

Ito K, Chen J, Seshan SV, Khodadadian JJ, Gallagher R, El Chaar M, Vaughan Jr ED, Poppas DP, Felsen D (2005). Dietary arginine supplementation attenuates renal damage after relief of unilateral ureteral obstruction in rats. *Kidney International* **68**: 515-528.

Jäättelä M (1999). Escaping cell death: survival proteins in cancer. *Experimental cell research* **248**: 30-43.

Johannesen J, Karlsen AE, Pociot F, Roenn SG, Nerup J (2003). Strain dependent rat iNOS promoter activity-correlation to identified WT-1 transcription factor binding site. *Autoimmunity* **36**: 167-175.

Kawaguchi M, Koshimura K, Murakami Y, Tsumori M, Gonda T, Kato Y (1999). Antihypertensive effect of insulin via nitric oxide production in the Zucker diabetic fatty rat, an animal model for non-insulin-dependent diabetes mellitus. *European Journal of Endocrinology* **140:** 341-349.

Kim YM, Bombeck CA, Billiar TR (1999). Nitric oxide as a bifunctional regulator of apoptosis. *Circulation Research* **84**: 253-256.

Kreidberg JA, Sariola H, Loring JM, Maeda M, Pelletier J, Housman D, Jaenisch R (1993). WT-1 is required for early kidney development. *Cell* **74**: 679-691.

Lane DP, Midgley C, Hupp T (1993). Tumour suppressor genes and molecular chaperones. *Philosophical transactions of the Royal Society of London. Series B, Biological sciences* **339**: 369-372.

Li F, Mao HP, Ruchalski KL, Wang YH, Choy W, Schwartz JH, Borkan SC (2002). Heat stress prevents mitochondrial injury in ATP-depleted renal epithelial cells. *American Journal of Physiology Cell Physiology* **283**: 917-926.

Liapis H (2003). Biology of congenital obstructive nephropathy. *Nephron. Experimental nephrology* **93**: 87-91.

Maheswaran S, Englert C, Bennett P, Heinrich G, Haber DA (1995). The WT1 gene product stabilizes p53 and inhibits p53-mediated apoptosis. *Genes Development* **9**: 2143-56.

Maheswaran S, Englert C, Zheng G, Lee SB, Wong J, Harkin DP, Bean J, Ezzell R, Garvin AJ, McCluskey RT, DeCaprio JA, Haber DA (1998). Inhibition of cellular proliferation by the Wilms tumor suppressor WT-1 requires association with the inducible chaperone Hsp70. *Genes Development* **12**: 1108-1120.

Manucha W (2007). Biochemical-molecular markers in unilateral ureteral obstruction. *Biocell* **31**: 1-12.

Manucha W, Carrizo L, Ruete C, Molina H, Vallés P (2005). Angiotensin II type I antagonist on oxidative stress and heat shock protein 70 (Hsp 70) expression in obstructive nephropathy. *Cellular and Molecular Biology* **51**: 547-555.

Manucha W, Oliveros L, Carrizo L Seltzer A, Vallés P (2004). Losartan modulation on NOS isoforms and COX-2 expression in early renal fibrogenesis in unilateral obstruction. *Kidney International* **65**: 2091-2107.

Manucha W, Vallés PG (2008). Cytoprotective role of nitric oxide associated with Hsp70 expression in neonatal obstructive nephropathy. *Nitric Oxide* **18**: 204-215.

Marcet-Palacios M, Davoine F, Adamko DJ, Moqbel R, Befus AD (2007). Human lymphocytes express the transcriptional regulator, Wilms tumor 1: the role of WT-1 in mediating nitric oxide-dependent repression of lymphocyte proliferation. *Biochemical and biophysical research communications* **363**: 283-287.

Mayo MW, Wang CY, Drouin SS, Madrid LV, Marshall AF, Reed JC, Weissman BE, Baldwin AS (1999). WT1 modulates apoptosis by transcriptionally upregulating the bcl-2 proto-oncogene. *EMBO Journal* **18**: 3990-4003.

Miyajima A, Chen J, Lawrence C, Ledbetter S, Soslow RA, Stern J, Jha S, Pigato J, Lemer ML, Poppas DP, Vaughan ED, Felsen D (2000). Antibody to transforming growth factor-beta ameliorates tubular apoptosis in unilateral ureteral obstruction. *Kidney International* **58**: 2301-2313.

Miyajima A, Chen J, Poppas DP, Vaughan Jr ED, Felsen D (2001). Role of nitric oxide in renal tubular apoptosis of unilateral ureteral obstruction. *Kidney International* **59**: 1290-1303.

Morrissey JJ, Klahr S (1999). Effect of AT_2 receptor blockade on the pathogenesis of renal fibrosis. *American Journal of Physiology* **276**: F39-F45.

Pritchard-Jones K, Fleming S, Davidson D, Bickmore W, Porteous D, Gosden C, Bard J, Buckler A, Pelletier J, Housman D (1990). The candidate Wilms tumor gene is involved in genitourinary development. *Nature* **346**: 194-197.

Scharnhorst V, Dekker P, van der Eb AJ, Jochemsen AG (2000). Physical interaction between Wilms tumor 1 and p53 proteins modulates their functions. *The Journal of biological chemistry* **275** (14): 10202-10211.

Schlaich MP, Oehmer S, Schneider MP, Delles C, Schmidt BM, Schmieder RE (2007). Effects of nitric oxide synthase inhibition and L-Arginine on renal haemodynamics in young patients at high cardiovascular risk, Atherosclerosis. **192**: 155-160.

Shepherd J, Hunninghake DB, Stein EA, Kastelein JJ, Harris S, Pears J, Hutchinson HG (2004). Safety of rosuvastatin. *American Journal of Cardiology* **94**: 882-888.

Takenaka IM, Leung SM, McAndrew SJ, Brown JP, Hightower LE (1995). Hsc70-binding peptides selected from a phage display peptide library that resemble organellar targeting sequences. *The Journal of Biological Chemistry* **270**: 19839-19844.

Topcu SO, Celik S, Erturhan S, Erbagci A, Yagci F, Ucak R (2008). Verapamil prevents the apoptotic and hemodynamic changes in response to unilateral ureteral obstruction. *International Journal of Urology* **15**: 350-355.

Trnka P, Hiatt MJ, Ivanova L, Tarantal AF, Matsell DG (2010). Phenotypic transition of the collecting duct epithelium in congenital urinary tract obstruction. *Journal of Biomedicine & Biotechnology* 696034. Epub 2009.

Vasil'eva TV, Michurina TV, Radionova IuV, Kuzin BA, Enikolopov GN, Khrushchov NG (1997). NO synthetase in the cells of different tissues and organs of the mouse. *Ontogenez* **28**: 458-462.

Xu Q, Hu Y, Kleindienst R, Wick G (1997). Nitric oxide induces heat-shock protein 70 expression in vascular smooth muscle cells via activation of heat shock factor 1. *Journal of Clinical Investigation* **100**: 1089-1097.

Yoo KH, Thornhill BA, Forbes MS, Chevalier RL (2010). Inducible nitric oxide synthase modulates hydronephrosis following partial or complete unilateral ureteral obstruction in the neonatal mouse. *American Journal of Physiology Renal Physiology* **298**: F62-F71.

Zhou MS, Schuman IH, Jaimes EA, Raij L (2008). Renoprotection by statins is linked to a decrease in renal oxidative stress, TGF-beta, and fibronectin with concomitant increase in nitric oxide bioavailability. *American Journal of Physiology Renal Physiology* **295**: F53-F59.