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ScienceDirect
Nitric Oxide xxx (2008) xxx–xxx
NITRIC OXIDE
Biology and Chemistry
www.elsevier.com/locate/yniox

Cytoprotective role of nitric oxide associated with Hsp70 expression in neonatal obstructive nephropathy

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Received 1 September 2007; revised 8 January 2008

Abstract

Nitric oxide (NO) has emerged as an important endogenous inhibitor of apoptosis. In this study, we postulated that the mechanism of apoptosis inhibition by NO would include stimulation of heat shock protein 70 (Hsp70) expression. Rats were subjected to unilateral ureteral obstruction (UUO) or sham operation, and kidneys were harvested 5 and 14 days after obstruction. After 14 days of obstruction, decreased endogenous NO and lower inducible nitric oxide synthase (iNOS) expression at mRNA and protein levels associated with downregulation of Hsp70 protein expression were shown in apoptosis induction, regulated by mitochondrial signal pathway, through the increased pro-apoptotic ratio Bax/BcL_2 and consequently caspase 3 activity. Conversely, 5 days after kidney obstruction, increased Hsp70 expression linked to increase NO and iNOS expression at transcriptional and post-transcriptional levels with absence of apoptotic response, were demonstrated. In obstructed neonatal rats, *in vivo* administration of L-Arginine induced heat shock protein 70 (Hsp70) expression, which was associated with cytoprotection from apoptosis and transiently decreased nicotinamide adenine dinucleotide phosphate reduced form (NADPH) oxidase activity. Opposite effects were obtained after nitro L-Arginine methyl ester (L-NAME) treatment. The interaction between B-cell lymphoma 2 anti-apoptotic members (BcL_2) and Hsp70 in the presence of L-Arginine and L-NAME, was determined by coimmunoprecipitation. Binding of BcL_2 and Hsp70 increased after L-Arginine administration. These findings suggest that NO can produce resistance to obstruction-induced cell death by mitochondrial apoptotic pathway, through the induction of Hsp70 expression, in neonatal unilateral ureteral obstruction.
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Keywords: Nitric oxide; BcL_2; Neonatal unilateral ureteral obstruction; Apoptosis; Caspase 3; Hsp70

The functional integrity of the kidney depends on the normal development as well as on the physiological cell turnover, apoptosis induction being essential for these mechanisms. Congenital obstructive nephropathy, a major cause of chronic renal failure in infancy, is characterized by decreased proliferation and increased apoptosis [1]. Programmed cell death leads to renal tubular atrophy and tubular loss in neonatal unilateral ureteral obstruction (UUO) [2]. Moreover, the severity of the apoptotic response to unilateral ureteral obstruction is far greater in the neonatal than in the adult rat, a factor that be likely contribute to the impaired growth of the obstructed development kidney [3].

Nitric oxide (NO) has been implicated in apoptosis for UUO, being a controversial key. Effects of NO in apoptosis depend on the dose, environment and/or redox state. Whereas excessive NO production induces cell death in several cell lines [4–7] conversely, protection against apoptosis had been shown in others [8,9]. Studies on the antiapoptotic mechanism of NO have identified NO target interactions that range from indirect to direct interaction with the apoptotic machinery. NO suppresses apoptosis through

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doi:10.1016/j.niox.2008.01.005

Please cite this article in press as: W. Manucha, P.G. Vallés, Cytoprotective role of nitric oxide associated with Hsp70 ..., Nitric Oxide (2008), doi:10.1016/j.niox.2008.01.005

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the direct caspase activity inhibition. Thiol group of caspase 3 (cys 163) susceptible to redox modification in the presence of NO can be efficiently *S*-nitrosylated [10] depending on the abundance of these molecules. Moreover, recent studies have proposed that B-cell lymphoma 2 anti-apoptotic member (BcL_2) cleavage can be inhibited by the caspase-3-like inhibitor Ac-DEVD-cho and/or NO, suggesting that the activated caspase-3-like proteases are responsible for the BcL_2 protein cleavage and the inactivation of the antiapoptotic function of BcL_2 [11]. In neonatal UUO, we have reported an apoptotic response through the pro-apoptotic regulation of the BcL_2 gene family and caspase 3 [12].

Due to the significant role of apoptosis in the pathogenesis of the renal cellular injury resulting from urinary tract obstruction, the factors regulating the renal apoptotic response have been evaluated. Stretching of the renal tubular cells by transmitted increased hydrostatic pressure can provide a powerful mechanical stimulus to apoptosis in the obstructed kidney [12,13]. Ischemia is another stimulus to apoptosis, and UUO induces a profound reduction in renal blood flow and impairment of autoregulation of renal blood flow [13,14]. Moreover, reactive oxygen species are known to reduce the threshold of tissues to undergo apoptosis [15], and reactive oxygen species are significantly increased in the chronically obstructed kidney [16]. The neonatal obstructed kidney may be particularly susceptible to the generation of reactive oxygen species, because endogenous renal antioxidant enzymes, including superoxide dismutase, are suppressed in the neonate [17].

Under normal physiological conditions, a balance between superoxide and nitric oxide exists *in vivo*.

NO and superoxide react together at a diffusion-controlled rate to yield peroxynitrite ($ONOO^-$), which inflicts cellular injury through oxidation of many biological molecules. Furthermore, $ONOO^-$ has also been implicated in the inactivation of Mn and Fe superoxide dismutase [18]. In contrast, NO may protect cells from reactive oxygen intermediate (ROI)-mediated cytotoxicity by scavenging superoxide anions which are implicated in toxicity through the formation of hydrogen peroxide or hydroxyl radical [19]. Nitric oxide has been shown to inhibit superoxide anion generation. The mechanism for such inhibition is thought to be due to the inactivation of nicotinamide adenine dinucleotide phosphate reduced form (NADPH) oxidase due to the scavenging effects of NO on superoxide [20].

Induction of the stress response includes synthesis of heat shock proteins (HSPs) that have been well characterized in cells injured from a variety of renal insults [21]. These proteins are generally classified into families according to their apparent molecular weight and respective inducers and play essential roles in protein chaperoning and cellular protection [22]. In addition, certain HSPs (including Hsp70) confer cellular protection by modulating the engagement and/or progression of apoptosis [23]. Recently, we have demonstrated that after 24 h of UUO, protection against tubulointerstitial fibrosis by Losartan, independent from changes in blood pressure, includes decreased oxidative stress linked to upregulation of Hsp70 expression [24].

In this study, we examined the consequences of NO on obstruction-induced apoptosis in renal cortex from neonatal UUO. We report that NO prevents obstruction-induced cell death by mitochondrial apoptotic pathway, through the induction of heat shock protein 70 (Hsp70).

Material and methods

Surgical procedure

Neonatal rats (Wistar Kyoto, males and females) were subjected to sham operation or complete UUO within the first 48 h of life. Under isoflurane, the abdomen was surgically opened by a left lateral incision, the left ureter was exposed and a 6.0 silk suture was used to place a ligature. The incision was closed in a single layer. The animals were allowed to recover from anesthesia and returned to their mothers. After 5 and 14 days of obstruction, animals were sacrificed with a lethal injection of pentobarbital and their obstructed and control kidneys were decapsulated, removed, and weighed. Successful ureteral ligation was confirmed at the time of kidney removal by observation of important hydronephrosis. Left kidney of sham group was also nephrectomized.

All the experimental procedures of this study have been previously approved by the Laboratory Animal Ethical Committee of the School of Medicine, Cuyo University, Mendoza (32/95 C.D.) The experiments were conducted in accordance with guidelines of the CEEA (Ethical Committee of Animal Experimentation of Argentina).

Identification of renal tubular cell apoptosis

TUNEL technique

Kidneys were then dehydrated, embedded in paraffin and serially sectioned (3–4 μm) on a microtome (Leica, Nussloch, Germany). Thereafter, kidney sections were deparaffined in xylene and rehydrated through graded ethanols to water. Endogenous peroxidase activity was quenched by incubation with 2% (v/v) H_2O_2 in phosphate buffer saline (PBS) for 5 min at room temperature (RT). Afterwards, staining and immunohistochemical techniques were performed.

After the digesting and quenching steps, equilibration buffer was applied directly to the sections for 5 min and working strength TdT enzyme (at a concentration of 1:5 in reaction buffer) was then applied directly for 1 h at 37 °C. A biotin-conjugated anti-digoxigenin antibody (Sigma) was used at 1:1500 dilution in PBS, pH 7.4, to incubate the tissue sections overnight at 4 °C. Then, the sections were incubated with biotinylated anti-mouse IgG (Dako, Carpinteria, CA, USA) at 1:100 dilution for 45 min at RT and later with peroxidase-labeled streptavidin (strept AB Complex/HRP, Dako) at 1:100 dilution for 45 min at RT. After a brief wash, 3,3′-diaminobenzidine tetrahydrochloride (0.5 mg/ml)/H_2O_2 (0.01%), a chromogen substrate was incorporated. Tissue sections were lightly counterstained with 0.5% with hematoxylin to reveal nuclei, and the slides were observed with a Zeiss Axioskop 2 microscope. For positive control, we used paraffin sections from involuting prostates of castrated rats ($n = 2$).

For the quantification of apoptotic epithelial cells, 10 consecutive fields were randomly selected in each renal cortex and they were evaluated at 400×, on a 10 × 10 grid, by using an image analyzer (Image Pro-Plus 4.0, 1998, Maryland, USA). Results were expressed as the number of apoptotic cells per mm^2.

RT-PCR and semiquantification of mRNA for iNOS, BcL_2 and Bax

Total ribonucleic acid (RNA) was obtained by using Trizol reagent (Gibco BRL). Two micrograms of total RNA were denatured in the pres-

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ence of 0.5 μg/50 μL Oligo $(dT)_{15}$ primer and 40 U recombinant ribonuclease inhibitor RNasin (Promega, USA). Reverse transcription was performed in the presence of mixture by using 200 units of Reverse Transcriptase M-MLV RT in reaction buffer, 0.5 mM dNTPs each, and incubated for 60 min at 42 °C. The copy deoxy nucleic acid (cDNA) (10 μL) was amplified by polymerase chain reaction (PCR) by standard conditions. Each cDNA aliquot was amplified (30 cycles) for inducible nitric oxide synthase (iNOS), BcL_2, BcL_2–associated X protein (Bax) and β-actin, (primers designed, Table 1).

Densitometric analysis was performed by using National Institutes of Health Image 1.6 software (Rasband Wayne et al., Division of Computer Research and Technology NIH, Bethesda).

The iNOS, BcL_2 and Bax signals were standardized against β-actin signal for each sample and results were expressed as a ratio.

Protein determination for iNOS, Hsp70, BcL_2 and pro-caspase 3

Cortex tissues were homogenized and protein concentrations were quantified by Bradford assay using bovine serum albumine (BSA) 10 mg/ml as a standard. Protein samples were prepared in sodium dodecyl sulphate (SDS) sample buffer (31.25 mM Tris–HCl, pH 6.8, 10% glycerol, 0.0025% bromophenol blue, 10 mM dithiothreitol (DTT), 1% SDS). A total of 20–50 μg of proteins were electrophoresed in 0.1% SDS and 8% polyacrylamide gel with 4% stacking gel and electrophoretically transferred to nitrocellulose. Gently removed blot from gel and placed it in a small plastic container containing about 10 ml of Ponceau S protein staining solution (to view extent of protein transfer or to ensure protein transfer) were performed.

Non-specific binding sites were blocked by incubating each membrane in 5% non-fat dry milk in PBS plus 0.1% Tween for 1 h at RT, washed, and then incubated overnight in the primary antibodies against iNOS (dilution 1:3000), Hsp70 (dilution 1:3000), BcL_2 (1:2000) and pro-caspase 3 (1:3000), from Sigma Chemical Co., Chemicon and Santa Cruz Biotechnology, respectively. Detection was accomplished with secondary antibodies (DAKO) and detected with enhanced chemiluminescence system (ECL, Amersham) and exposure to X-ray film (Amersham).

Densitometric analysis was carried out by image analysis software, the photographs were digitalized by using a scanner (LACIE Silver Scanner for Macintosh) and the Desk Scan software (Adobe Photo Shop) on a desktop computer. Densitometric analysis was performed using the US National Institute of Health Image 1.66 software (Rasband Wayner et al., Division of Computer Research and Technology NIH, Bethesda, USA). The magnitude of the immunosignal was standardized to 1 for the corresponding control renal tissue values.

Table 1
Primers designed from rat sequences for RT-PCR

Primer	Sequence	Annealing (°C)	Predicted product size, (bp)
iNOS			
Antisense	5′-GCTTCTGGTCGATGT CATGAGCAA-3′	55	222
Sense	5′-GCATGGACCAGTATA AGGCAAGCA-3′		
BcL_2			
Antisense	5′-CTTGTGGCCCAGGTA TGC-3′	59	708
Sense	5′-ATGGCGCAAGCCGG GAGAA-3′		
Bax			
Antisense	5′-TCAGCCCATCTTCTT CCAGAT-3′	59	550
Sense	5′-ATGGACGGGTCCGG GGAGC-3′		
β-Actin			
Antisense	5′-GTGCCACCAGACAG CACTGTGTTG-3′	65	201
Sense	5′-TGGAGAAGAGCTAT GAGCTGCCTG-3′		

Caspase 3 activity assay

The activity of caspase 3 was determined by using the CaspACE™ Assay System (Promega, Madison, WI, USA).

Aliquots of cytosolic homogenates (37.5 μL) were diluted in caspase assay buffer (312.5 mM *N*-2-hydroxyethyl-piperazine-*N*′-2-ethanesulfonic acid HEPES, pH 7.5, 31.25% sucrose, 0.3125% CHAPS (3-(3-cholamidopropyl)-dimethyl ammonio)-1 propane-sulfonate) + dimethylsulphoxide (DMSO) 2 μL + DTT 100 mM 10 μL, and incubated for 30 min at 37 °C. After the addition of 2.5 mM of the substrate (CPP32 Substrate Ac-Asp-Glu-Val-Asp-7-amido-4-methyl coumarin (Ac-DEVD-AMC), incubation was performed for 60 min at 37 °C. Peptide cleavage was measured over 1 h at RT by using an spectrofluorometric fluorescent plate reader (Fluoro Count TM; AF10001, Cambers Company, USA) at a wavelength of 360 nm excitation and 460 nm emission. Specific caspase 3 activity was expressed as pmol of AMC liberated/min/μg protein. A reversible aldehyde inhibitor (CPP32 inhibitor Ac-DEVD-CHO) was used as negative control.

Determination of nitrite levels in homogenates from renal cortex

We measured nitrite levels from renal cortex kidney, as previously described [25] with minor modifications. Homogenates from renal cortex tissue of obstructed and control kidneys were incubated with 10 mmo1/L L-Arginine in a buffer (pH 7.40) containing 25 mmol/L HEPES, 140 mmol/L NaCl, 5.4 mmol/L KCl, 1.8 mmol/L $CaCl_2$, 1 mmol/L $MgCl_2$, and 5 mmol/L glucose at 37 °C for 24 h. After centrifugation at 6400 rpm for 20 min, the supernatants were used for the assay of NO production and the amount of NO_2^- was corrected by means of the protein amount, measured according to the Bradford method. Nitrite was measured by a spectrophotometer at 540 nm wavelength by using the Griess reaction. The NO_2^- present was expressed as nmol of nitrite generated per μg protein.

NADPH oxidase and superoxide dismutase activity assays

Cellular injury from oxidative stress occurs when reactive oxygen species (ROS) accumulate in excess on the host defense mechanisms. The NADPH oxidase activity is one of the parameters highly involved in the apoptosis induction because it is anion superoxide producing.

NADPH oxidase activity was measured by luminol technique. Luminol (5-amino-2,3-dihydro-1,4-phthalazine SIGMA) is widely used as a chemioluminescence reagent [26].

Samples were homogenized and centrifuged at 6000 rpm for 30 min. The supernatant was separated and again centrifuged to 19,500 rpm and the protein concentration of the membrane fraction lysate was determined with the Bradford protein assay (Bio-rad, Hercules, CA, USA).

Samples (100 μL) of the membrane fraction re-suspended in lysis buffer were rapidly read in the spectrofluorometer (Fluoro Count TM; AF10001, Cambers Company, USA) in order to establish the basal value of each sample. Then, 2 μL of β-NADPH (β-nicotinamide adenine dinucleotide phosphate, reduced form, SIGMA) 0.1 mmol/L and 2 μL of Luminol 5 μmol/L in DMSO were incorporated and they were read during 10 min (360 nm excitation and 460 nm emission). The values were expressed as relative fluorescence units by micrograms of protein and per minute of incubation.

Spectrophotometric assay for superoxide dismutase (SOD) activity was performed [27]. The assay is based on the SOD-mediated increase in the rate of autoxidation of 5,6,6α,11β-tetrahydro-3,9,10 trihydroxybenzo[*c*]fluorene (BXT-01050) in aqueous alkaline solution. This autoxidation yields a chromophore with a maximal absorbance wavelength of 525 nm. The SOD activity was determined from the V_s/V_c ratio of the autoxidation rates measured

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in the presence (V_s) and in the absence (V_c) of sample. One SOD activity unit (U-525) has been defined as the activity that doubles the autoxidation background ($V_s/V_c = 2$).

"In vivo" administration of L-Arginine and L-NAME in UUO neonatal rats: effects on apoptosis induction

In order to state if the mechanism of apoptosis inhibition by NO would include stimulation of Hsp70, nitro L-Arginine methyl ester L-NAME (50 mg/kg/day) [28], L-Arginine (100 mg/kg/day) [29] or deionized water (vehicle) were administrated by oral gavage for 14 days to neonatal rats previously subjected to sham operation or complete UUO within the first 48 h of life.

The cortex from pretreated 14 day obstructed and control rats, was chopped in tiny pieces with razor blade and it was homogenized with a dounce style tissue homogenizer on ice (buffer: 250 mM; sucrose, 20 mM Tris–base, 5 mM ethylenediaminetetraacetic acid (EDTA), pH 7.4 protease inhibitors were added: Soybean Tripsin inhibitor 5 μg/ml and phenylmethylsulphonyl fluoride (PMSF) 0.01%. After, centrifugation at 7000 rpm (6000*g*) 15 min at 5 °C was performed.

In the supernatant, the measurement of nitrites, caspase 3 activity, Bax/BcL_2 mRNA expression and iNOS. NADPH oxidase activity, BcL_2 protein levels and Hsp70 protein levels were performed.

Hsp70 antibody

To evaluate the participation of Hsp70 upon apoptosis induction in neonatal UUO, cortex homogenates from obstructed kidney (OK) and control kidney (CK) were incubated in the presence and in the absence of anti-Hsp70 antibody. In the supernatant (cytosolic fraction) Hsp70 protein expression and caspase 3 activity were evaluated in the same fraction.

BcL_2 immunoprecipitation—Hsp70 coprecipitation

To evaluate the interaction between BcL_2 and Hsp70 related to the nitric oxide bioavailability, coimmunoprecipitation was performed in obstructed and control cortex homogenates from rats previously treated with L-Arginine (100 mg/kg/day), responsible for NO production and a NO inhibitor L-NAME (50 mg/kg/day) for 14 days.

Table 2
Kidney weight/Body weight (mg/g) in obstructed and sham-operated rats

Days of obstruction	CKW/BW	OKW/BW
5	7.23 ± 0.48	8.47 ± 1.00
14	6.52 ± 0.20	5.85 ± 0.20[a]

After 5 and 14 days of obstruction, ratio of obstructed kidney weight/body weight (OKW/BW) mg/g and control kidney weight/body weight ratio (CKW/BW) mg/g are shown. Values are means ± SEM ($n = 8$).
[a] OKW/BW vs CKW/BW following 14 days of obstruction, $^{*}p < 0.05$.

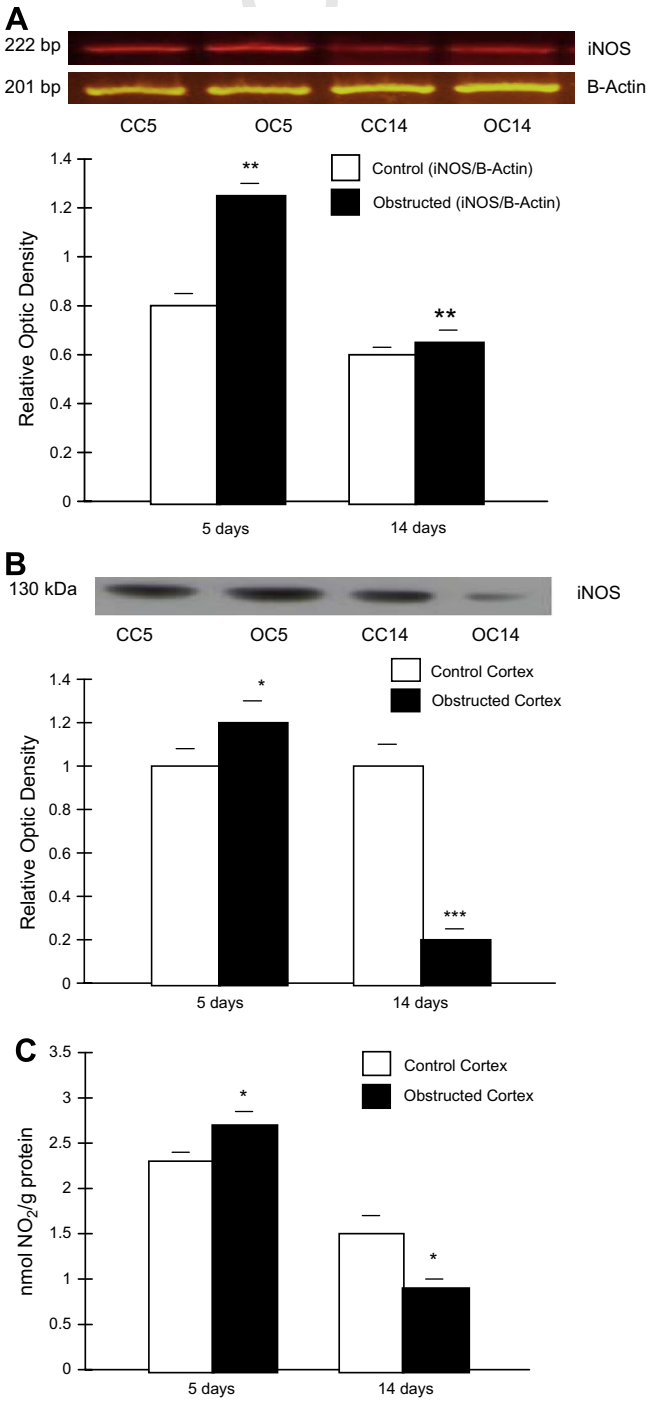

Fig. 1. Endogenous NO generation and iNOS expression in kidney cortex after 5 and 14 days of UUO. (A) Representative gel of iNOS mRNA in control and obstructed cortexes kidney after 5 days of obstruction, and in control and obstructed kidney for 14 days. Housekeeping gene β-actin expression is shown in the line herebelow, in the same order as the densitometry bars. Graphical representation of iNOS/β-actin mRNA ratio showed an increase expression of iNOS isoform in obstructed cortex (OC) vs control cortex (CC) $^{**}p < 0.01$ after 5 days of obstruction. Decreased iNOS expression from 14 days OC vs 5 days OC was demonstrated $^{**}p < 0.01$. Results are means ± SEM of six independent observations. (B) Representative Western blot and densitometric analysis of iNOS protein levels from cortex kidneys after 5 days of obstruction and following 14 days of obstruction. Immunoblots were quantified for iNOS expression. The relative amount of iNOS protein was determined after normalization of the level of iNOS protein of the appropriate control: 1 and was shown in histograms beneath the corresponding blots. Intensive decreased in iNOS protein levels from kidneys obstructed for 14 days compared to CC $^{***}p < 0.001$. Light increase of iNOS protein levels in OC compared to CC after 5 days of obstruction $^{*}p < 0.05$. Results are means ± SEM of six separate experiments. (C) Measurement of nitrite generated (nmol NO_2 generated/μg protein) Following 14 days of obstruction decreased NO in OC vs CC $^{*}p < 0.05$, was shown. After 5 days of obstruction, increased NO in OC vs CC $^{*}p < 0.05$ was shown. Decreased NO in 14 days OC vs 5 days OC $^{***}p < 0.001$.

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The coimmunoprecipitation was carried out using the Dynabeads M-280 Tosylactivated (Dynal, Biotech). A concentration of 3 μg antibody/10^7 Dynabeads was used. Briefly, the antibody BcL_2 was dissolved in a 0.1 M borate buffer, pH 9.5, added to the Dynabeads and then vortexed for 1 min. After 48 h incubation, rotating at 4 °C, sample were placed in the magnet and the supernatants were removed and discarded. The coated beads were washed three times with a buffer containing PBS, pH 7.4 (phosphate buffered saline) with 0.1% BSA one time and another containing 0.2 M Tris, pH 8.5 with 0.1% BSA. Subsequently, equal volumes of homogenate supernatants, adjusted to contain equal quantities of protein were added to the coated beads. Following a 1 h incubation rotating at 2–8 °C, samples were placed in the magnet and the supernatants were removed and discarded. The beads were washed three times by using a 0.1 M Na-phosphate, pH 7.4 and were suspended in an equal volume of 2× sample buffer, and boiled for 3 min. The supernatant was removed and stored at −70 °C. Samples were boiled for 3 min before Western blotting. The Hsp70 level was standardized against BcL_2 level for each experimental condition, and results were expressed as a ratio.

Statistical analysis

The results were assessed by one-way analysis of variance for comparisons among groups.

Differences among groups were determined by Bonferroni post-test.

A $p < 0.05$ was considered to be significant. Results are given as means ± standard error medium (SEM).

Statistical tests were performed by using GraphPad In Sat version 3.00 for Windows 95 (Graph Pad Software, Inc., San Diego, CA, USA).

Results

Kidney weight/body weight ratio

As shown in Table 2, after 5 days of obstruction there were no differences in kidney weight/body weight ratio from OK related to left kidneys of the control group (CK). Decreased kidney weight/body weight ratio from the animals following 14 days of obstruction was demonstrated when it was compared to the one of the control group ($n = 8$).

In vivo—apoptosis induction—is associated with diminished NO

Western blot analysis of obstructed kidney cortex for 14 days revealed decreased iNOS protein expression as compared with control: 0.2 ± 0.05 vs 1 ± 0.8, $p < 0.001$; $n = 6$ (Fig. 1B). Lower nitrite generation (nmol NO_2^-/μg protein) in obstructed cortex (OC) related to control: 0.90 ± 0.10 vs 1.50 ± 0.20, $p < 0.05$, $n = 6$ (Fig. 1C) and decreased mRNA iNOS expression in OC for 14 days compared with OC for 5 days: 0.60 ± 0.03 vs 1.25 ± 0.05, $p < 0.01$, $n = 6$ was shown (Fig. 1A). Linked to decreased NO generation, apoptosis induction-dependent on intracellular mitochondrial pathway was shown in the same fraction in 14 days obstructed cortex compared with control through the decreased anti-apoptotic gen BcL_2 expression 0.30 ± 0.03 vs 0.58 ± 0.01, $p < 0.01$ and increased pro-apoptotic ratio Bax/BcL_2 1.40 ± 0.10 vs 0.85 ± 0.15, $p < 0.05$, $n = 6$, respectively, (Fig. 2A). Western blot analysis demonstrated an intensive decrease in 32 kDa pro-caspase 3 protein expression due to its cleavage to an active protein 0.20 ± 0.05 vs 1.10 ± 0.05,

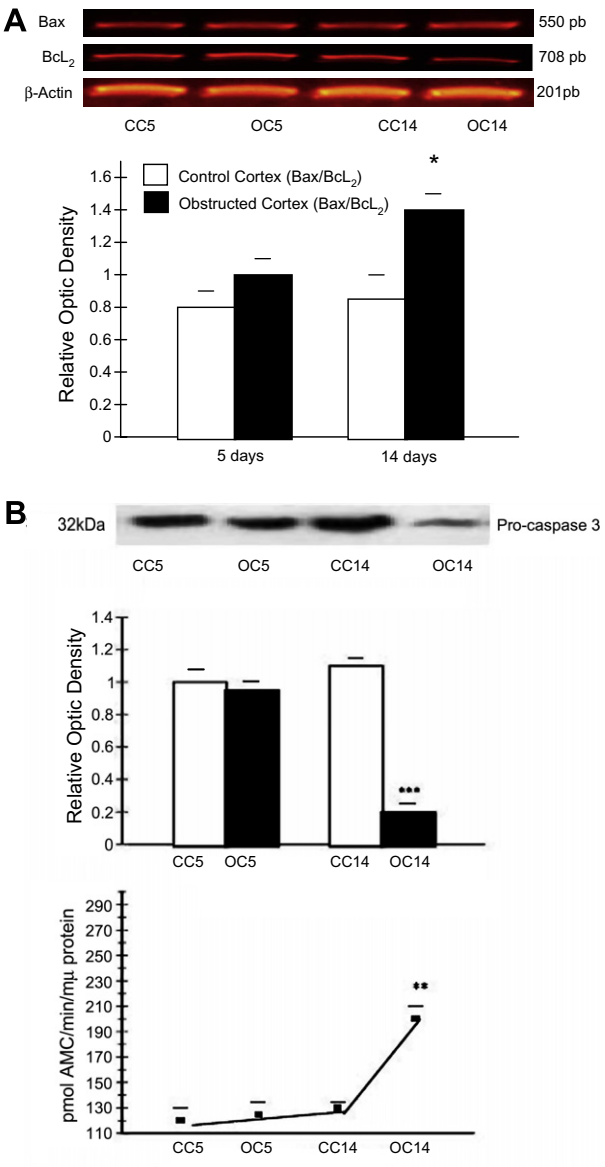

Fig. 2. Mitochondrial apoptotic pathway induction after 5 and 14 days of kidney obstruction. (A) Induction of mRNA expression for BcL_2 and Bax and the ratio of mRNA Bax/mRNA BcL_2 in kidney cortexes after UUO for 5 and 14 days. Representative gels of BcL_2 and Bax mRNA in cortex from OK and CK are shown. The corresponding housekeeping β-actin is included in below. Histograms show the relative concentration of mRNAs for BcL_2 and Bax to β-actin mRNA. Cortexes obstructed for 14 days compared with CC $^{*}p < 0.05$. Data represent means ± SEM of six independent experiments. (B) Western blot analysis for 32 kDa pro-caspase 3 protein and caspase 3 activity in obstructed and control cortex kidneys. Upper panel: Total protein (50 μg) was extracted and equal amounts of protein were loaded and separated by molecular weight on 12% SDS–polyacrylamide gel electrophoresis (PAGE). Blot represents one out of six separate experiments. Densitometric analysis of pro-caspase 3 protein has shown decreased protein level in 14 days OC vs CC $^{***}p < 0.001$, $n = 6$. Lower panel: Caspase 3 activity was assessed by level of Ac-DEVD-AMC cleavage release of fluorescence AMC tag. Activity is expressed as pmol AMC/min/μg protein. Cortexes obstructed for 14 days compared with CC $^{**}p < 0.01$. Caspase 3 activity and pro-caspase 3 protein assay data were obtained from the same six independent samples.

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$p < 0.001$, $n = 6$, as determined by an increase in caspase 3 activity pmol AMC/min/μg protein, 200 ± 10 vs 130 ± 5, $p < 0.01$, $n = 6$, in 14 days obstructed cortex compared with control (Fig. 2B). Increased number of apoptotic cells in collecting ducts (CD) was shown exceeding the one in the proximal tubules (PT) (Fig. 3). A ninefold higher apoptotic cells were shown in CD from 14 days obstructed kidney related to that measured in controls: 135 ± 15 vs 15 ± 5, $p < 0.001$, $n = 6$.

Conversely, increased iNOS mRNA expression 1.25 ± 0.05 vs 0.80 ± 0.05, $p < 0.001$, $n = 6$; higher iNOS protein levels 1.2 ± 0.01 vs 1 ± 0.1, $p < 0.05$, $n = 6$ and nitrites levels as a marker of NO generation (nmol NO_2^-/μg protein); 2.70 ± 0.15 vs 2.30 ± 0.10, $p < 0.05$, $n = 6$, were revealed in kidney cortex after 5 days of obstruction compared to control. Kidney obstruction for 5 days did not show significant differences neither in the pro-apoptotic ratio Bax/BcL_2 expression (Fig. 2A) nor in pro-caspase 3 protein levels and caspase 3 activity (Fig. 2B). No significant increase in the number of apoptotic cells per mm^2 in cross-sectioned CD and PT from kidney cortexes was shown (Fig. 3).

Effect of time obstruction on Hsp70 protein expression

To determine if NO was associated with Hsp70 expression, Western blotting was performed in cortex kidneys. After 5 and 14 days of obstruction we verified an increase in Hsp70 protein expression in day 5 in obstructed cortex compared with control: 1.58 ± 0.08 vs 1 ± 0.05, $p < 0.001$, $n = 6$. On the contrary, after 14 days of obstruction, no signal was detected on Hsp70 protein expression (Fig. 4). During this period of obstruction a high apoptotic index as well as a significant caspase 3 activity were shown.

NADPH oxidase and SOD activity

After 5 days of obstruction, light increase on NADPH oxidase activity (fluorescence units/μg protein/min) was observed related to control cortex: 2000 ± 150 vs 2500 ± 100, $n = 4$, $p < 0.05$. After 14 days of obstruction, intensive increase on NADPH oxidase activity was shown related to control: 7776 ± 74 vs 3000 ± 120, $p < 0.001$, $n = 4$, (Fig. 5). Decreased superoxide dismutase activity

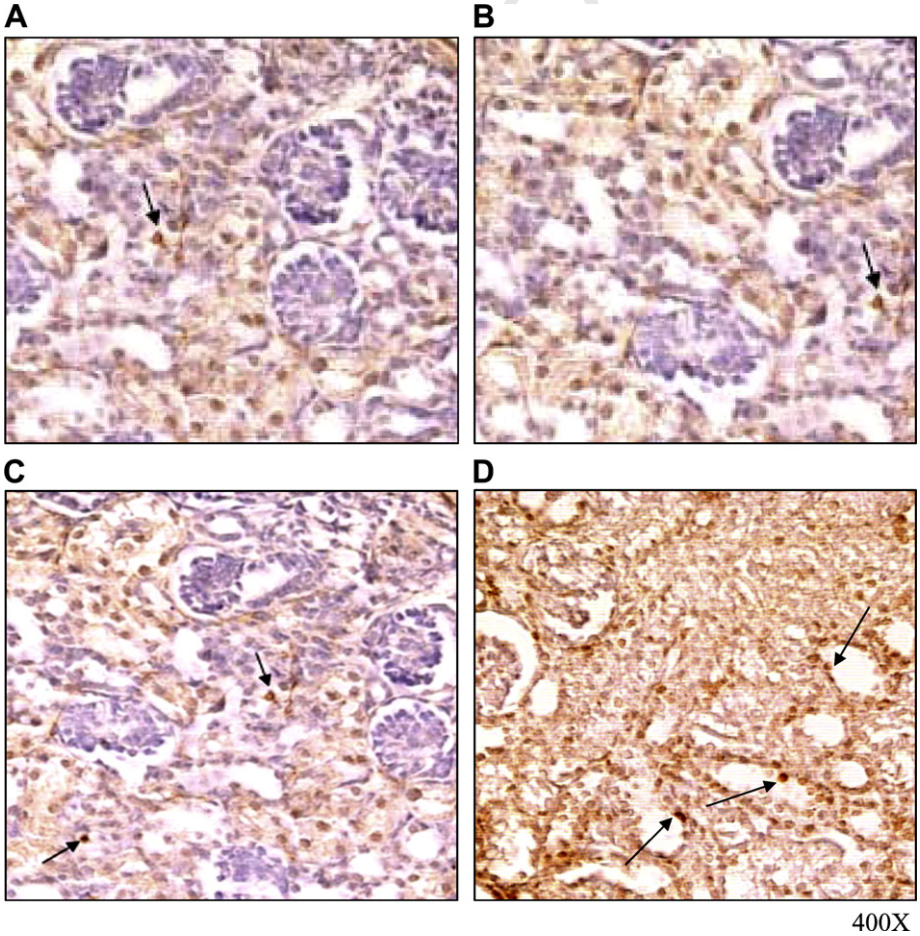

Fig. 3. Histologic sections of neonatal kidney following unilateral obstruction for 5 and 14 days. Apoptotic nuclei localization by TdT-uridine-nick-end-labeling technique: apoptotic nuclei appear as heavy brown-staining nuclei in tubule epithelial cells (arrows). (A and B) Five days control and obstructed kidney cortexes. Apoptotic cells are rarely seen in CD and PT. (C) Fourteen days control kidney cortex. Apoptotic cells are scarcely seen in CD and PT. (D) Apoptotic nuclei appear as heavy brown-staining nuclei in dilated collecting ducts and in lesser proportion in proximal tubules, 14 days after ipsilateral obstruction in cortex kidney, Magnification 400×.

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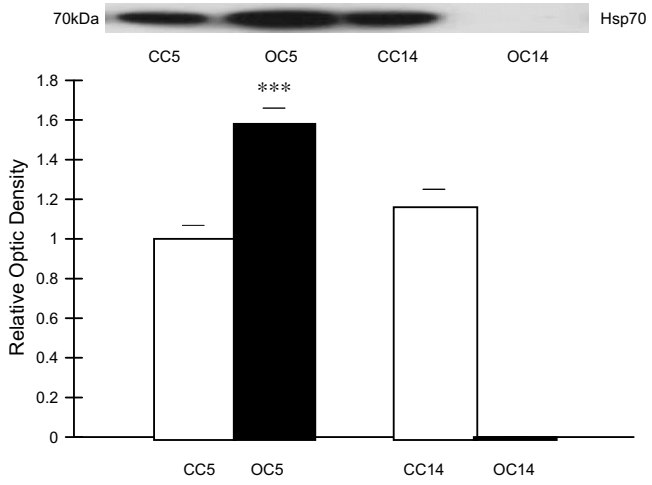

Fig. 4. Increased Hsp70 expression during early unilateral kidney obstruction in neonatal rats. Western blot analysis of Hsp70 from cortex kidney following 5 and 14 days of obstruction. The relative amount of Hsp70 protein was determined after normalization of the level of Hsp70 protein of the appropriate control. Densitometric analysis of Hsp70 protein abundance showed a significant increase on Hsp70 expression in OC vs CC, $^{***}p < 0.001$ after 5 days of obstruction. No signal was detected on Hsp70 protein expression after 14 days of obstruction. Data represent means ± SEM of six separate experiments.

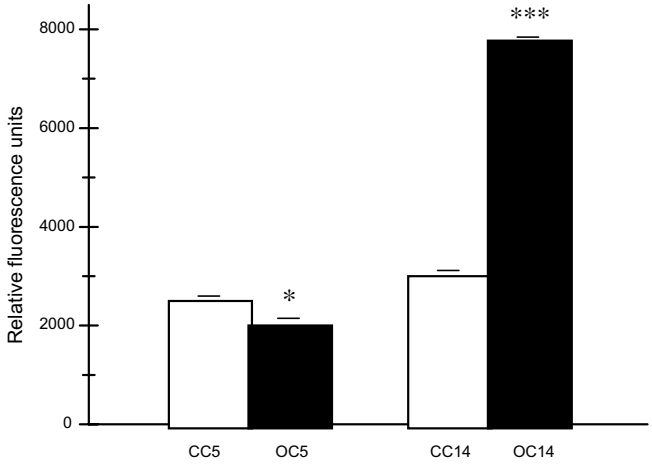

Fig. 5. Effect of obstruction for 5 and 14 days on NADPH oxidase activity in cortex kidney. NADPH oxidase activity was measured by a chemioluminescence assay. Increased NADPH oxidase activity was shown after 14 days of obstruction OC vs CC $^{***}p < 0.001$. Slight decreased NADPH oxidase activity was shown after 5 days OC vs CC $^{*}p < 0.05$. Each bar represents the mean ± SEM of four separate experiments.

were demonstrated following 14 days of obstruction (U/mL) 10 ± 4 vs 60 ± 5, $p < 0.001$, $n = 4$. No differences were noticed on the antioxidant level after 5 days of kidney obstruction $= 40 \pm 8$ vs 52 ± 7, $n = 4$.

"In vivo" administration of L-Arginine and L-NAME in UUO neonatal rats: effects on apoptosis induction

We examined the *in vivo* effect of L-Arginine (100 mg/kg/day) and L-NAME (50 mg/kg/day) pretreatment on the apoptotic response induction, in control and obstructed cortex homogenates.

In vivo administration of L-Arginine for 14 days showed higher endogenous NO (nmol/μg protein): 8.4 ± 0.45 vs 3.2 ± 0.3, $n = 4$, $p < 0.001$, increased iNOS: 1.2 ± 0.05 vs 1 ± 0.05, $p < 0.05$, $n = 4$, (Fig. 6A and B) and higher Hsp70 protein levels: 1.9 ± 0.06 vs 1 ± 0.09, $p < 0.01$, $n = 4$ in control cortex homogenates (Fig. 7). Since our data showed that L-Arginine induced Hsp70 expression, we next examined whether treatment with L-Arginine in the same control cortex homogenate, could result in resistance to apoptosis induction. Fig. 8A, showed that apopto-

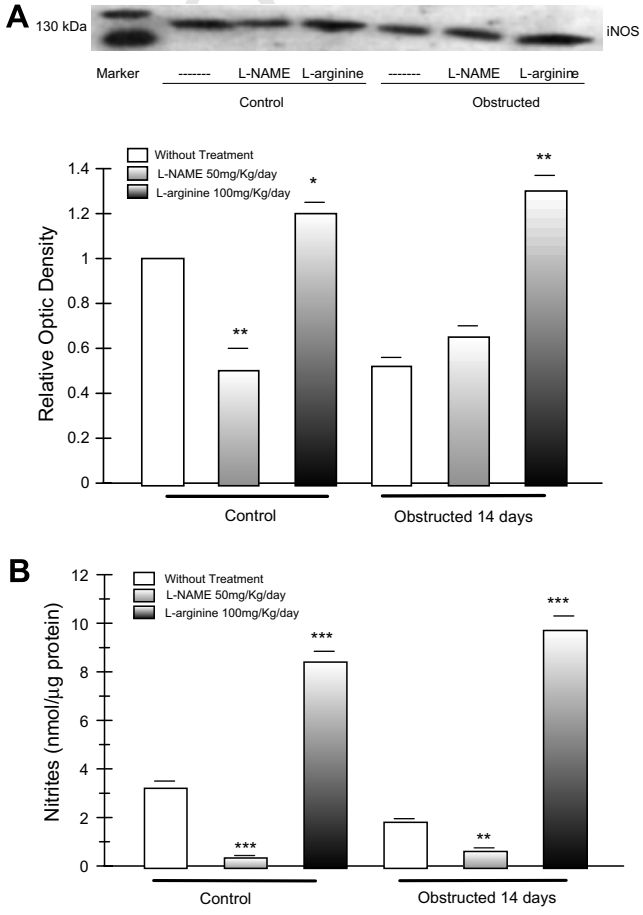

Fig. 6. *In vivo* effect of L-Arginine and L-NAME treatment for 14 days, on endogenous NO levels and iNOS protein expression. (A) Representative Western blot and densitometry of iNOS in control and 14 days obstructed cortex homogenate from rats pretreated with L-Arginine or L-NAME control and obstructed cortexes from neonatal rats pretreated with L-Arginine resulted in higher iNOS protein levels related to non-treated rats, $^{*}p < 0.05$ and $^{**}p < 0.01$, respectively. On the contrary, control cortex homogenates from animals treated with L-NAME for 14 days, showed lower iNOS abundance vs non-treated $^{**}p < 0.01$. The relative amount of iNOS protein was determined after normalization of the level of iNOS protein of the appropriate control: 1. (B) Measurement of nitrite generated (nmol NO_2 generated/μg protein) control and obstructed cortexes from neonatal rats pretreated with L-Arginine resulted in higher NO levels related to non-treated rats, $^{***}p < 0.001$ both. Nitrite levels were nearly completely inhibited after L-NAME treatment. Each bar represents the mean ± SEM of four separate experiments.

sis was almost completely inhibited. Absence of the apoptotic index (1.52 ± 0.03 vs 1.00 ± 0.01, $n = 4$) as well as decreased caspase 3 activity (10 ± 1 vs 15 ± 2, $n = 4$) were shown (Fig. 8C). Overproduction of reactive oxygen intermediate (ROI) has previously been identified as a key component of apoptotic pathways. Therefore, after L-Arginine treatment lower NADPH oxidase activity was shown: 1000 ± 120 vs 2500 ± 100, $p < 0.001$, $n = 4$ (Fig. 9).

Obstructed kidney homogenates from rats pretreated with L-Arginine, showed higher endogenous NO (nmol/μg protein): 9.7 ± 0.6 vs 1.8 ± 0.15, $n = 4$, $p < 0.001$, increased iNOS: 1.3 ± 0.07 vs 0.52 ± 0.04, $n = 4$, $p < 0.01$ (Fig. 6B and A) and detectable Hsp70 protein levels (Fig. 7). Obstructed kidney homogenates from L-Arginine pretreated rats compared to the one from rats without L-Arginine administration, showed decreased apoptotic index Bax/BcL_2 (0.9 ± 0.02 vs 1.90 ± 0.20, $p < 0.01$, $n = 4$) (Fig. 8A), increased BcL_2 protein expression (Fig. 8B) and a light decrease in the caspase 3 activity (17 ± 3 vs 23 ± 3, $n = 4$) (Fig. 8C). Lower NADPH oxidase activity was demonstrated in 14 day obstructed homogenates from L-Arginine pretreated rats compared to the obstructed kidney homogenate from rats without treatment (3000 ± 119 vs 6000 ± 120, $p < 0.001$, $n = 4$) (Fig. 9).

Conversely, treatment of control rats with L-NAME showed lower iNOS: 0.5 ± 0.1 vs 1 ± 0.05, $p < 0.01$, $n = 4$, decreased endogenous NO(nmol/μg protein): 0.33 ± 0.10 vs 3.2 ± 0.3, $p < 0.001$, $n = 4$ (Fig. 6A and B) and detected no signal of Hsp70 expression in cortex (Fig. 7).

Increased Bax/BcL_2 apoptotic index (2.50 ± 0.02 vs 1.00 ± 0.01, $p < 0.001$, $n = 4$) (Fig. 8A), as well as increased caspase 3 activity (40 ± 5 vs 15 ± 2, $p < 0.001$, $n = 4$) (Fig. 8C) and higher NADPH oxidase activity (4500 ± 89 vs 2500 ± 100, $p < 0.001$, $n = 4$) (Fig. 9), were demonstrated when control kidneys from L-NAME treated rats were compared to non-treated control rats.

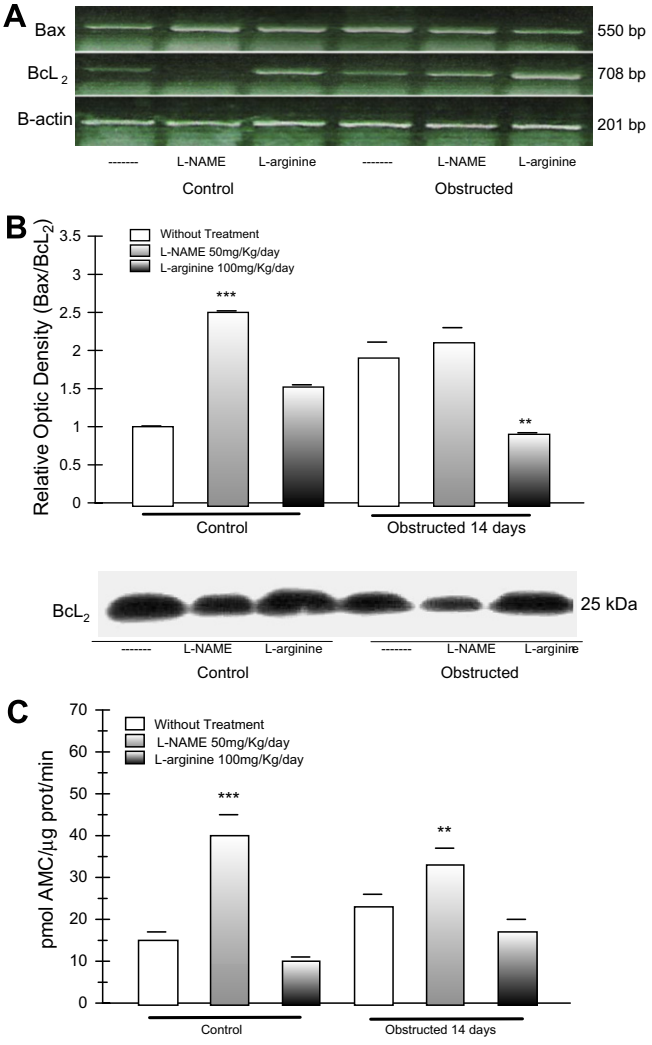

Fig. 8. *In vivo* effect of L-Arginine and L-NAME treatment on apoptosis induction. (A) mRNA expression for Bcl2 and Bax and the ratio of mRNA Bax/mRNA Bcl2. (B) Representative Western blot of BcL_2. (C) Caspase 3 activity was assessed by level of Ac-DEVD-AMC cleavage release of fluorescence AMC tag. Activity is expressed as pmol AMC/min/μg protein. Control and 14 day obstructed cortex homogenates from L-Arginine pretreated neonatal rats resulted in resistance to apoptosis induction. After L-Arginine for 14 days, absence of apoptotic index, increased BcL_2 expression as well as caspase 3 activity near control non-treatment were shown. Conversely, L-NAME treatment of control rats showed increased Bax/BcL_2 apoptotic index as well as increased caspase 3 activity, compared to non-treated control rats, $^{***}p < 0.001$ and $^{***}p < 0.001$, respectively. After 14 days of obstruction, pretreated rats with L-NAME increased caspase 3 activity compared to the one of the non-treated animals $^{**}p < 0.01$. Each bar represents the mean ± SEM of four separate experiments.

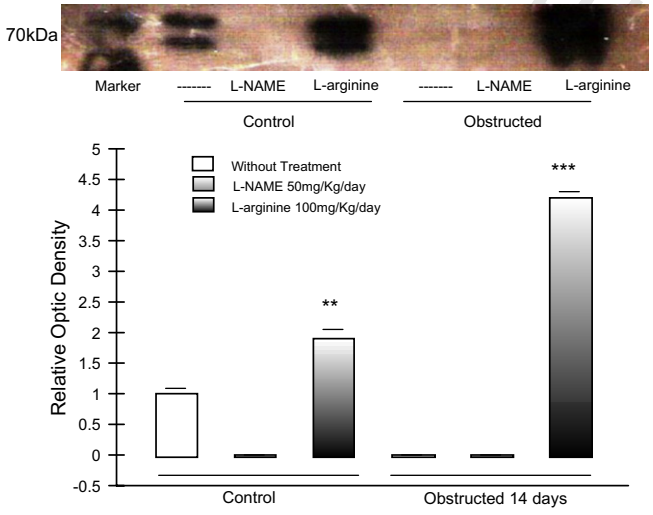

Fig. 7. *In vivo* effect of L-Arginine and L-NAME treatment on Hsp70 protein expression in neonatal rats. Representative Western blot and densitometry of Hsp70 in control and 14 days obstructed renal cortex homogenate from rats pretreated with L-Arginine or L-NAME. After L-Arginine-pre treatment for 14 days, a significant increase on inducible Hsp70 protein levels was noted in obstructed and control cortex homogenates related to cortex tissue of non-treated rats, $^{***}p < 0.01$ and $^{**}p < 0.001$, respectively. Absence of Hsp70 expression was demonstrated after L-NAME treatment. Blot represents one out of four separate experiments.

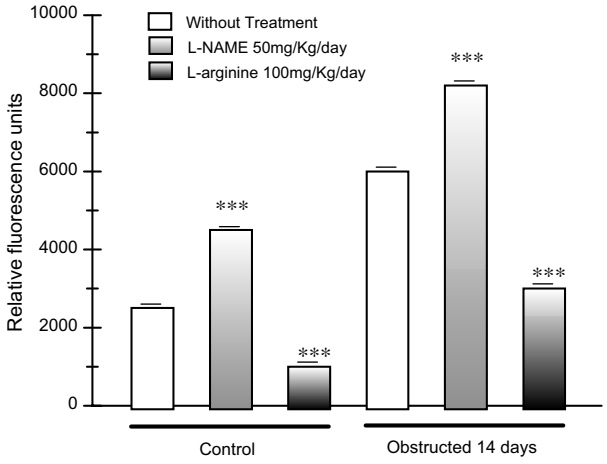

Fig. 9. *In vivo* effect of L-Arginine and L-NAME treatment on NADPH activity. After *in vivo* L-Arginine treatment lower NADPH oxidase activity was shown in control and 14 days renal obstructed homogenates compared to homogenates of non-treated rats, $^{***}p < 0.001$, both. Conversely, L-NAME treatment significantly increased NADPH oxidase activity in control and 14 days obstructed homogenates compared to homogenates of non-treated rats, $^{***}p < 0.001$, both. Each bar represents the mean ± SEM of four separate experiments.

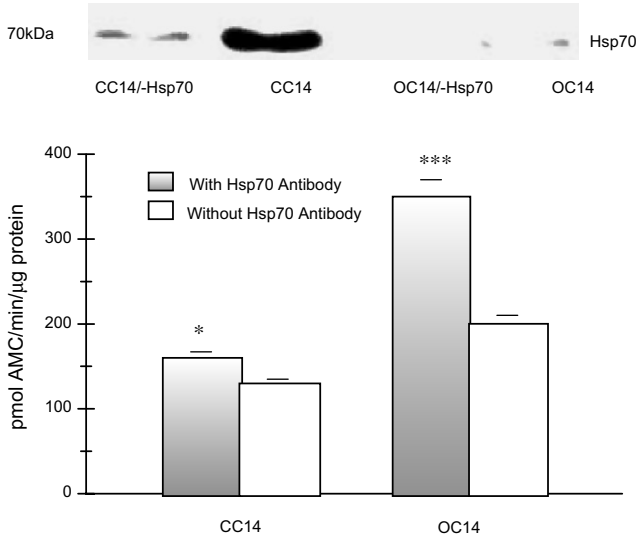

Fig. 10. Cortex fractions from 14 days obstructed and control kidney incubated in the presence or in the absence of the antibody against Hsp70. Top panel: Representative Western blot and densitometry of Hsp70 demonstrating the effects of anti-Hsp70 antibody in control and 14 days obstructed cortexes. Lower panel: Significant increase of caspase 3 activity was shown in control and obstructed cortex in the presence of the antibody against Hsp70: $CK^{Anti\text{-}Hsp70\ Ab}$ vs CK, $^{*}p < 0.05$, $OK^{Anti\text{-}Hsp70\ Ab}$ vs OK, $^{***}p < 0.001$.

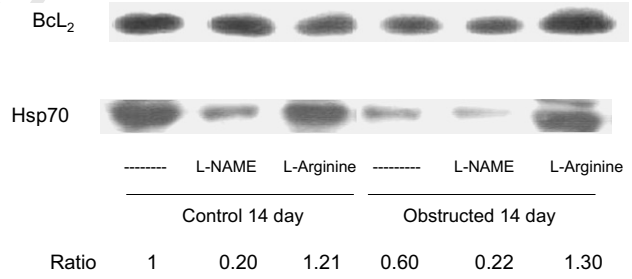

Fig. 11. Representative coimmunoprecipitation of BcL_2 and Hsp70. Cortex tissues from 14 days obstructed and control kidney rats pretreated with a NO inducer L-Arginine and with a NO inhibitor L-NAME for 14 days. Cortex tissues were immunoprecipitated with BcL_2 antibody and were coprecipitated and analyzed for Hsp70. The amount of Hsp70 coprecipitating with BcL_2 was expressed as a ratio. Higher ratio between both proteins was shown in control cortex and obstructed cortex after L-Arginine administration, related to homogenate from control rats pretreated only with the buffer.

Obstructed kidney homogenates from L-NAME pretreated rats, showed lower endogenous NO (nmol/µg protein): 0.6 ± 0.15 vs 1.8 ± 0.15, $p < 0.01$, $n = 4$; no changes in iNOS protein expression and no detectable Hsp70 expression (Figs. 6B and 7). These changes were associated with absence of difference in the apoptotic index Bax/BcL_2 (2.10 ± 0.20 vs 1.90 ± 0.20, $n = 4$), (Fig. 8A), decreased BcL_2 protein expression (Fig. 8B), increased caspase 3 activity (33 ± 4 vs 23 ± 3, $p < 0.01$, $n = 4$) (Fig. 8C) and higher NADPH oxidase activity (8200 ± 115 vs 6000 ± 120, $p < 0.001$; $n = 4$) (Fig. 9).

Hsp70 involvement on apoptosis induction

We have next evaluated the Hsp70 involvement on apoptosis induction through the incubation of cortex homogenates in the presence or absence of anti-Hsp70 antibody. As seen in Fig. 10, increased caspase 3 activity (AMC liberated/minute/µg protein) was shown in control and obstructed cortex in the presence of the antibody against Hsp70: $CK^{Anti\text{-}Hsp70\ Ab}$ vs CK, 160 ± 7 vs 130 ± 5, $p < 0.05$, $n = 4$; $OK^{Anti\text{-}Hsp70\ Ab}$ vs OK, 350 ± 20 vs 200 ± 10, $p < 0.001$, $n = 4$, respectively.

BcL_2 immunoprecipitation—HSP70 coprecipitation

To further evaluate the interaction between BcL_2 and Hsp70 in the presence of an NO inducer and NO inhibitor, coimmunoprecipitation was performed. Homogenates from 14 days obstructed and control kidney rats, previously treated with L-Arginine and L-NAME for 14 days were immunoprecipitated with BcL_2 antibody and analyzed to evaluate the presence of coprecipitating protein Hsp70. Interaction of BcL_2 and Hsp70 was observed under control and experimental conditions. After L-Arginine treatment, an increased amount of Hsp70 coprecipitated with BcL_2 expressed as a ratio of control incubated only in the presence of medium were shown with a percentage increase of 21% and 30% in control cortex and 14 days obstructed cortex, respectively, $n = 4$ (Fig. 11).

Discussion

Apoptosis induction plays a fundamental role in the morphogenesis and in the renewal of the cells in this tissue. The effects of the messenger diffusible molecule NO on

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apoptosis might be a NO level dependence and motive of permanent controversies because of its bifunctional role. UUO renal tubular apoptosis is related to renal tubular atrophy and renal tissue loss [30,31]. The study we present here provides evidence for cytoprotective effect of NO linked to Hsp70 against the mitochondrial apoptosis pathway in early neonatal obstruction.

In the present study, we have shown that NO protects cortex tubular epithelial cells from obstructed cortex kidney-induced cytotoxicity and apoptosis. Studies after 5 and 14 days of unilateral kidney obstruction revealed that induction of Hsp70 protein occurred in parallel to protection from obstruction-induced apoptosis.

Both pro-apoptotic and anti-apoptotic effects of NO have been demonstrated so far [32]. The capacity of NO to induce apoptosis was first appreciated by Albina et al., who confirmed that NO-dependent death of murine peritoneal macrophages activated by interferon-γ (IFN-γ) and lipopolysaccharide (LPS) is mediated through apoptosis [33]. Notwithstanding, more recent studies have shown an anti-apoptotic effect of NO. Mannick et al. have shown that endogenous iNOS expression or exposure to low doses of NO donors inhibited apoptosis in human B lymphocytes [34]. Since then, similar findings have been reported *in vitro* and *in vivo* [35,36]. From *in vitro* studies in stretched epithelial cells and *in vivo* studies in obstructed kidney of iNOS−/− mice support for an anti-apoptotic role to NO have been provided [37].

The question of whether NO promotes or inhibits apoptosis has been quite controversial, multiple mechanism for the inhibition of apoptosis by NO may exist in a single cell.

In our study, downregulation of Hsp70 protein expression associated with decreased endogenous NO and lower iNOS at the level of gene expression and protein expression were shown in the induction of apoptosis following 14 days of obstruction. A temporal relationship was shown between 14 days obstruction and apoptosis regulated by mitochondrial signal pathway, through the increased pro-apoptotic ratio Bax/BcL_2 and, consequently caspase 3 activity. Conversely, increased Hsp70 expression linked to increased NO and iNOS expression at transcriptional and post-transcriptional levels with absence of apoptotic tubular cell response were shown after obstruction for 5 days. These results suggest that the presence of NO linked to Hsp70 protein expression may serve to modulate apoptotic process in obstructed kidney. Hsp70 induction is an early survival signal elaborated by stressed cells to counter cellular damage and hasten recovery [38]. This chaperone is known to bind to nascent and immature proteins, and to prevent premature and improper binding and folding. Hsp70 also confers cellular protection by modulating the engagement and/or progression of apoptosis [23]. Evidence to support the hypothesis that apoptosis was associated with decreased NO joined to lower Hsp70 protein expression was also established herein by *in vivo* manipulation of endogenous NO. Control cortex of L-NAME pretreated rats resulted in lower levels of Hsp70 and iNOS protein expression with downregulation of BcL_2 at the level of gene expression and protein expression together with increased caspase 3 activity. The cellular effects of apoptosis were reversed by L-Arginine treatment.

Moreover, to further demonstrate the association of NO with Hsp70 in the apoptotic response, interaction between Hsp70 and BcL_2 in the presence of an NO inhibitor and NO inducer was performed. An antibody directed against BcL_2 was used to precipitate native BcL_2 protein. Coprecipitation of both proteins increased to 21% in control homogenates from rats pretreated with a NO inducer related to control rats pretreated with buffer. The mechanism by which NO stimulates the expression of Hsp70 may involve the interaction of NO with thiol-containing molecules. NO readily oxidizes the most abundant low molecular weight thiol glutathione, forming *S*-nitrosothiols and disulfide. This action stimulates the Hsp70 which protect cells from apoptotic cell death. [39]. In a previous report, pretreatment of hepatocytes with NO altered redox state accompanied by oxidation of glutathione (GSH) and formation of *S*-nitrosoglutathione (GSNO), both being involved in Hsp70 mRNA induction [8]. In our study we have demonstrated that the apoptotic effect by lower NO-mediated decreased Hsp70 expression was associated with the direct induction of apoptotic signal transduction involving the activation of caspase 3 by decreasing stabilization of BcL_2. Given its BcL_2 localization within mitochondria and its role in preventing cytochrome *c* release, preservation of BcL_2 by Hsp70 could account for the protection of epithelial cells [40]. Nevertheless, Hsp70 may intervene at several points to halt progression of the apoptotic cascade. Hsp70 may act by preventing cell death by interfering with the ability of cytocrome *c* and Apaf-1 to recruit pro-caspase-9. Hsp70 therefore suppressing apoptosis by directly associating with Apaf-1 and blocking the assembly of a functional apoptosome [41]. Production of ROS has been identified as a key component of apoptotic pathways involving activation of endogenous endonucleases [42] and direct DNA fragmentation [43]. In our study, after 14 days of obstruction, oxidative stress was elevated through the increased NADPH oxidase activity as well as decreased superoxide dismutase activity. These results lead to a pronounced increase in total oxidant activity in obstruction. Conversely, NADPH oxidase activity was transiently suppressed when rats were pretreated with L-Arginine, similar to the results obtained after 5 days of kidney obstruction.

Higher renal endogenous NO levels in obstructed kidneys for 5 days or after L-Arginine pretreatment, induced Hsp70 expression, which has been shown to have anti-apoptotic or cytoprotective effects. Thus, it is likely that Hsp70 expression by L-Arginine administration protected the cells from early obstruction-mediated apoptosis and cytotoxicity. These results allow us to suggest that upregulation of Hsp70 and increased endogenous NO may be an early line of defense to cytoprotect cortex tubule cells in

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early kidney obstruction. Hsp70 expression induction precedes conventional markers of renal injury.

In the present study we did not explore the mechanisms involved in ROS production inhibition by L-Arginine-induced Hsp70 expression. Previously, it has been suggested that Hsp70 may block signal transduction to the mitochondria, resulting in the inhibition of mitochondrial reactive oxygen intermediate (ROI) production by inhibiting either second lipid messenger(s) to mitochondria [44]. Alternatively, it has also been possible that Hsp70 may enhance the chaperon-mediated import of precursor proteins into mitochondria which control mitochondrial function [45,46] leading to decreased ROS formation. Pretreatment with the NO-generating compound *S*-nitroso, *N*-acetylpenicillamine (SNAP) have been shown to protect cultured rat hepatocytes from tumoral necrosis factor alfa (TNFα)-induced cytotoxicity and apoptosis through the stimulation of Hsp70 expression [8].

Taken together, our data demonstrate that the effect of NO interacting with Hsp70 is a result of the capacity of both to prevent mitochondrial apoptotic pathway in neonatal early kidney obstruction. Induction of Hsp70 protects cells not only from damage due to apoptosis induction but also from damage due to oxidative injury. These findings demonstrated that NO can induce cytoprotection in early obstructed kidney cortex tubular epithelial cells, through the stimulation of Hsp70 expression.

Acknowledgments

This work was performed with financial support from the Research and Technology Council of Cuyo University (CIUNC) from Mendoza, Argentina/No. 631/02 and from CONICET, PICT/2005 No. 05-33827 to P.G. Vallés.

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Please cite this article in press as: W. Manucha, P.G. Vallés, Cytoprotective role of nitric oxide associated with Hsp70 ..., Nitric Oxide (2008), doi:10.1016/j.niox.2008.01.005