

# The Nrf2–Keap1 cellular defense pathway and heat shock protein 70 (Hsp70) response. Role in protection against oxidative stress in early neonatal unilateral ureteral obstruction (UUO)

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Received: 2 July 2010 / Revised: 4 August 2010 / Accepted: 5 August 2010 / Published online: 24 August 2010  
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**Abstract** Perturbation of renal tubular antioxidants and overproduction of reactive oxygen species may amplify the proinflammatory state of renal obstruction, culminating in oxidative stress and tubular loss. Here, we analyzed the heat shock protein 70 (Hsp70) response and the function and signal transduction of NF-E2-related protein 2 (Nrf2) transcription factor on oxidative stress modulation in obstruction. Rats were subjected to unilateral ureteral obstruction or sham operation and kidneys harvested at 5, 7, 10, and 14 days after obstruction. Hsp70 expression and Nrf2 activity and its downstream target gene products were assessed. After 10 and 14 days of obstruction, enhanced lipid peroxidation through higher thiobarbituric acid reactive substances levels and increased oxidative stress resulted in reduced total antioxidant activity and enhanced nicotinamide adenine dinucleotide phosphate reduced

(NADPH) oxidase activity were demonstrated. This was accompanied by decreased inducible Hsp70 expression and a progressive reduction of nuclear Nrf2 and its target gene products glutathione S-transferase A2 (GSTA2) and NADPH/quinone oxidoreductase 1 (NQO1), whereas the Nrf2 repressor Kelch-like ECH-associated protein-1 (Keap1) was upregulated. By contrast, on early obstruction for 7 days, lack of increased oxidative markers associated with higher inducible Hsp70 protein levels and a rapid nuclear accumulation of Nrf2, Keap1 downregulation, and mRNA induction of the identified Nrf2-dependent genes, NQO1 and GSTA2, were shown. For these results, we suggest that the magnitude of cytoprotection in early obstruction depends on the combined contribution of induced activation of Nrf2 upregulating its downstream gene products and Hsp70 response. Impaired ability to mount the biological response to the prevailing oxidative stress leading to renal injury was shown in prolonged obstruction.

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**Keywords** Hsp70/72 · Nrf2–Keap1 · Oxidative stress ·  
Unilateral ureteral obstruction

## Abbreviations

ABTS	2,2'-Azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)
AEBSF	4-(2 Aminoethyl)-benzenesulfonyl fluoride
ARE	Antioxidant response elements
CAT	Catalase
CC	Control cortex
CD	Directive Council
CEEA	Ethical Committee of Animal Experimentation of Argentina

CLC	Contralateral cortex
COX-2	Cyclooxygenase-2
GPx	Glutathione peroxidase
GSTA2	Glutathione S-transferase A2
HDAC1	Histone deacetylase 1
HO-1	Heme oxygenase-1
Hsp	Heat shock protein
IL-1	Interleukin-1
IL-6	Interleukin-6
Keap1	Kelch-like ECH-associated protein-1
L-	L-Arginine methyl ester
NAME	
NADPH	Nicotinamide adenine dinucleotide phosphate reduced
NO	Nitric oxide
NOS	Nitric oxide synthase
NQO1	NADPH/quinone oxidoreductase 1
Nrf2	NF-E2-related nuclear factor erythroid-2
OC	Obstructive cortex
ROS	Reactive oxygen species
SOD	Superoxide dismutase
TAA	Total antioxidant activity
TBARS	Thiobarbituric acid reactive substances
TNF- $\beta$	Tumor necrosis factor $\beta$
UO	Unilateral ureteral obstruction
8-	8-Hydroxy-2'-deoxyguanosine
OHdG	

## Introduction

Congenital obstructive nephropathy, the major cause of chronic renal failure in infancy and childhood (Warady 1997), is characterized by impaired renal growth and development and a reduction in nephron number (Chevalier 1995). Genetic and nongenetic factors responsible for the lesions are largely unidentified, and attention has been focused on minimizing obstructive renal injury (Chevalier et al. 2010). The cellular and molecular events responsible for obstructive injury to the developing kidney have been elucidated from animal models (Matsell and Tarantal 2002). Tubular injury/dysfunction as a result of mechanical injury in obstruction elicit a cascade of proinflammatory events including angiotensin, transforming growth factor- $\beta$  and adhesion molecules that culminate in oxidant stress and tubular loss (Ricardo and Diamond 1998). Thus, mediators of cellular mechanisms initiated by ischemia and subsequent reperfusion are involved in reactive oxygen species (ROS) generation. The neonatal obstructed kidney is particularly susceptible to the generation of reactive oxygen species because endogenous renal antioxidant enzymes, including superoxide dismutase, are suppressed in the neonate (Gupta

et al. 1999). Perturbation of renal tubular antioxidants and overproduction of ROS may amplify the proinflammatory state of renal obstruction, culminating in tubulointerstitial injury and fibrosis (Klahr and Morrissey 1998).

Through reactive metabolites, oxidative stress alters cellular signaling, promoting apoptosis and the activation of stress response pathways, including the antioxidant, endoplasmic reticulum stress, DNA damage, and heat shock responses (Jacobs and Marnett 2007). Heat shock protein (Hsp) induction is a cytoprotective mechanism that enhances cell survival in the wake of thermal or chemical stress. A principal function of Hsps is to chaperone other proteins, binding to nascent polypeptide chains as well as to unfolded and damaged proteins (Hahn and Li 1982; Howard et al. 1993). Previously, we have reported that nitric oxide can produce resistance to obstruction-induced cell death by mitochondrial apoptotic pathway, through the induction of heat shock protein 70 (Hsp70) expression, in neonatal unilateral ureteral obstruction (Manucha and Vallés 2008).

In addition to heat shock, reactive metabolites also activate the antioxidant response, an adaptive mechanism to defend against oxidative stress that is regulated by the transcription factor, NF-E2-related nuclear factor erythroid-2 (Nrf2; Chen et al. 2005). Under unstimulated conditions, Nrf2 is sequestered in the cytoplasm, where it is associated with Kelch-like ECH-associated protein-1 (Keap1), an actin-binding protein (Jaiswal 2004; Lee et al. 2003). The presence of a stimulus leads to the disruption of the Nrf2–Keap1 complex and nuclear translocation of Nrf2; Nrf2 then accumulates and localizes to the nucleus where it heterodimerizes with specific cofactors, including members of the Maf protein family, and coordinates upregulation of cytoprotective genes through the initiation of transactivation at antioxidant response elements (AREs) within the regulatory regions of these genes (Kobayashi and Yamamoto 2005, 2006; Leonard et al. 2006; Itoh et al. 1997). An imbalance between production of ROS and antioxidant defense appears to be involved in the pathogenesis of diverse parenchymal diseases.

In this study, we will investigate the temporal induction of both Hsp70 activation and Nrf2-dependent antioxidant genes as coordinated adaptive cytoprotective mechanisms on the oxidative stress modulation in neonatal kidney obstruction.

## Material and methods

### Surgical procedure

Neonatal rats (Wistar Kyoto, males and females) were subjected to sham operation or complete unilateral ureteral

obstruction (UUO) within the first 48 h of life. Under isofluorane, 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. Pups recovered on a warm surface and were returned to their mothers. After 5, 7, 10, and 14 days of obstruction, animals were sacrificed with a lethal injection of pentobarbital, and their kidneys were removed, decapsulated, and weighed. Successful ureteral ligation was confirmed at the time of kidney removal by observation of important hydronephrosis. Left kidney of sham group (control) 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 CD). The experiments were conducted in accordance with guidelines of the Ethical Committee of Animal Experimentation of Argentina.

#### *Immunohistochemical studies*

Kidney paraffin sections (5–6  $\mu\text{m}$  thickness) were dewaxed in xylol, rehydrated, and incubated with 3%  $\text{H}_2\text{O}_2$  for 30 min to quench endogenous peroxidase activity. After washing in Tris-buffered saline (0.05 M Tris-HCl, 0.15 M NaCl), pH 7.6, and non-specific blocking with 10% bovine serum albumin for 30 min at room temperature, the sections were immunostained to reveal Hsp70. A commercial immunoperoxidase kit was used (Dako EnVision System, Dako Corporation, Carpinteria, CA, USA). The antibody applied was a mouse monoclonal antibody (BRM-22) against the inducible and constitutive forms of Hsp70 (Sigma Chemical Co, St. Louis, MO, USA). The positive reaction was evaluated considering the specific location of immunostaining (renal structure and cell compartment: nucleus, cytoplasm, membrane) and the intensity of the immunoreaction. Negative controls included tissues unexposed to primary antibodies as well as tissues exposed to control immunoglobulin G. Positive controls were human breast cancer biopsy samples.

#### *Total antioxidant activity*

Total antioxidant activity (TAA) was measured by an improved ABTS radical cation decolorization assay applicable to both lipophilic and hydrophilic antioxidants (Re et al. 1999). The preformed radical monocation of 2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) ( $\text{ABTS}^+$ ) was generated by oxidation of 7 mM ABTS with 2.45 mM potassium persulfate and was reduced in the presence of such hydrogen-donating antioxidants. The influences of both the concentration of antioxidant and duration of reaction on the inhibition of the radical cation

absorption were taken into account when determining the antioxidant activity. Percent inhibition was determined as: % inhibition =  $[(A_o - A_f)/A_o] \times 100$ , where  $A_o$  was the absorbance at 734 nm of the unscavenged radical cation solution while  $A_f$  was the absorbance after the addition of the antioxidant sample. Absorbance was monitored for 6 min after the addition of the antioxidant solution. All measurements were made in triplicate at each concentration of the standard and solvent samples, with an appropriate solvent blank being used for each assay.

#### *Nicotinamide adenine dinucleotide phosphate reduced oxidase activity assay*

Cellular injury from oxidative stress occurs when reactive oxygen species (ROS) accumulate in excess on the host defense mechanisms, the nicotinamide adenine dinucleotide phosphate reduced (NADPH) oxidase activity is one of the parameters highly involved because it is anion superoxide producing. NADPH oxidase activity was measured by lucigenin-derived chemiluminescence assay. Samples were homogenized and centrifuged at 6,000 rpm for 30 min. The supernatant was separated and again centrifuged at 19,500 rpm and the protein concentration of the membrane fraction lysate was quantified by Lowry assay by using BSA as a standard. Sample (40  $\mu\text{L}$ ) of the membrane fraction were incubated in buffer Jude–Krebs, then  $\beta$  NADPH (SIGMA) was added (as substrate) and chemiluminescence was measured continuously for 3 min on a microplate fluorometer (Fluoroskan Ascent, Lab Systems, FL, USA). The values were expressed as relative luminescence units per micrograms of protein and per minute of incubation.

#### *Lipid peroxidation assay*

Thiobarbituric acid reactive substances (TBARS) were quantified as an index of lipid peroxidation with a colorimetric method modified by (Buege and Aust 1978). Frozen samples (500  $\mu\text{L}$ ) were mixed with 1 mL work solution (15% trichloroacetic acid, 0.25 N hydrochloric acid, 0.67% w/v thiobarbituric acid, 2.5 mM 1,1-butylated hydroxytoluene (BHT) solution, and 0.1 mL of 8.1% SDS) followed by 30 min heating at 95°C; pH value of the analytical reaction mixture was about 0.9 BHT was used to prevent lipid peroxidation during heating. After cooling either incubation, the chromogen was extracted with *n*-butanol and read spectrophotometrically at 532 against a reaction mixture “blank” lacking plasma but subjected to the entire procedure and extracted with *n*-butanol. To correct for background absorption, absorbance values at 572 nm were subtracted from those at 532 nm, the latter representing the absorption maximum of the 2:1 TBA/

MDA adduct. A molar extinction coefficient of 154,000 was used.

#### *Tissue preparation: nuclear fraction isolation*

The isolated cortexes were placed in ice-cold isolation buffer containing 0.5 M saccharose, 10 mM Tris-HCl, 1.5 mM MgCl<sub>2</sub>, 10 mM KCl, 10% glycerol, 1 mM EDTA, 1 mM DTT, 2 µg/mL aprotinin, 4 µg/mL leupeptin, 2 µg/mL chymostatin, 2 µg/mL pepstatin, and 100 µg/mL 4-(2-aminoethyl)-benzenesulfonyl fluoride, pH 7.4, and were homogenized by using a Dounce style tissue homogenizer. The homogenate was centrifuged at 4,000×g 5 min at 4°C to remove incompletely homogenized fragments and nuclei. The pellet was resuspended in lysis buffer and centrifuged at 12,000×g for 20 min at 4°C. Then, the supernatant was resuspended in isolation buffer and the aliquots (nuclear fractions) were saved at -70°C.

#### *Western blot analysis*

Protein concentrations were determined using Bradford protein assay (Bio-Rad). For Western blot, equal amounts of protein were resolved by SDS-PAGE, transferred into a 0.2 µm nitrocellulose membrane (GE Healthcare Amersham), and incubated in blocking buffer (20 mM Tris-HCl, pH 7.6, 140 mM NaCl, 0.05% Tween 20, 5% nonfat dry milk) prior to the addition of primary antibody. Following incubation with primary and secondary antibodies, the bound antibody was then visualized by using enhanced chemiluminescence (ECL) detection (GE Healthcare Amersham) and an analyzer of gels was used (LAS-4000 Luminiscent Image analyzer, Fujifilm Life Science). Densitometric quantification of the proteins band was done by using NIH image analysis software.

β-Tubulin western blot was done to normalize the cytosol and histone deacetylase 1 (HDAC1) to normalize nuclear fraction protein amount. Primary antibodies were obtained from the following sources: Hsp70 and β-tubulin from Sigma-Aldrich; Nrf2, Keap1, and HDAC1 from Santa Cruz Biotechnology; and all biotinylated secondary antibodies were purchased from Dako Cytomation.

#### *RT-PCR and semiquantification of mRNA for NADPH/quinone oxidoreductase 1, glutathione S-transferase A2, and β-actin*

Total RNA was obtained by using Trizol® reagent (Invitrogen). Two micrograms of RNA were denatured in the presence of 0.5 µg/50 µL Oligo (dT)<sub>15</sub> 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 cDNA (10 µL) was amplified by polymerase chain reaction by standard conditions. Each sample was measured for glutathione S-transferase A2 (GSTA2) and NADPH/quinone oxidoreductase 1 (NQO1) and β-actin (primers designed in Table 1).

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

#### *Statistical analysis*

The results were assessed by two-way ANOVA 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. Statistical tests were performed by using GraphPad In Sat version 5.00 for Windows Vista (GraphPad Prism Software, Inc, San Diego, CA, USA).

## **Results**

### Characteristics of animals

As shown in Table 2, after 5 and 7 days of unilateral obstruction, there were no differences in kidney weight/body weight ratio (KW/BW) between the animals within UUO or sham-operation group (control group). After 10 and 14 days of obstruction, the KW/BW from the neonatal rats subjected to UUO was lower than that of their counterparts in the control group, while the contralateral KW/BW was greater than that of the control group; see Table 2.

### Oxidative stress and lipid peroxidation markers at varying time intervals following UUO in the neonatal rat

Oxidative stress was evaluated by using analyses for total antioxidant activity and pro-oxidant NADPH oxidase activity this last; one of the key generators of superoxide in the kidney. TBARS products were also evaluated as diagnostic index of lipid peroxidation and peroxidative tissue injury.

Strong total antioxidant activity was shown in control cortex. UUO resulted in reduced total antioxidant activity from day 5 and 7 after obstruction, with a significant twofold reduction after 10 days and a very significant near threefold reduction of control after 14 days of UUO. There were no significant changes in total antioxidant activity between control and contralateral cortex after 14 days (Fig. 1).

By contrast, NADPH oxidase activity was significantly enhanced in 10- and 14-day obstructed cortex homogenates

**Table 1** Primers designed from rat sequences for RT-PCR

Primer	Sequence	Annealing (°C)	Cycles	Size (bp)
NQO1				
Sense	5'-ATC CTG GAA GGA TGG AAG-3'	55	35	97
Anti-sense	5'-CCT GCC TGG AAG TTT AGG-3'			
GSTA2				
Sense	5'-TGG AGA TTG ATG GGA TGA AG-3'	49	35	105
Anti-sense	5'-CAA TCA GGG CTC TCT CCT TC-3'			
β-actin				
Sense	5'-TGG AGA AGA GCT ATG AGC TGC CTG-3'	65	35	201
Anti-sense	5'-GTG CCA CCA GAC AGC ACT GTG TTG-3'			

related to controls with a twofold increase of cortex tissue after 14 days of obstruction related to control. No differences were shown between contralateral cortex homogenates related to controls (Fig. 2).

A clear increase in the obstructed kidney's TBARS concentration, a marker of lipid peroxidation was observed with a significant twofold increase of control after 10 days of UUO and a higher twofold and a half of control after 14 days of UUO. The contralateral (unobstructed) kidney showed no significant rise in TBARS levels compared to controls (Fig. 3).

#### Hsp70 protein expression in obstruction

Figure 4 shows the expression of Hsp70 in a control, obstructed, and contralateral kidney cortexes after 5, 7, and 14 days of UUO.

In 5-day obstructed cortex, Hsp70 staining was present in epithelial cell cytoplasm from the cortical collecting ducts (CCDs) and proximal tubules (PTs; Fig. 4b). Strong Hsp70 immunoreaction in the membrane and cytoplasm of the same tubular epithelial cells was shown after 7 days of obstruction (Fig. 4e). In cross-sections of epithelial cells from CCD and PT from contralateral and control kidney cortexes weak Hsp70 immunoreaction were shown after 5 and 7 days of obstruction (Fig. 4c, f). After 14 days of obstruction, decreased Hsp70 immunostaining was observed

in the cytoplasm from epithelial cell of PT and CCDs (Fig. 4h), opposite to this, Hsp70 overexpression was shown in the same epithelial duct segments from contralateral 14-day UUO (Fig. 4i).

#### Cytoprotective Hsp70 response in obstruction

We hypothesized that inducible Hsp72 activation was involved on the regulation of oxidative stress in obstruction. Cytosol preparations from renal cortex were used for western blot analysis. Antibody against Hsp70 protein recognized two bands, at 70 and 72 kDa. Figure 5 showed both the constitutive (Hsp70) and inducible form (Hsp72) of the protein. Increased inducible form of the protein, Hsp72 was demonstrated in cortex cytosol from kidneys obstructed for 5 and 7 days compared with control ( $22.3 \pm 2.14\%$   $n=4$ ,  $p<0.01$  and  $31.8 \pm 3.17\%$   $n=4$ ,  $p<0.001$ ), respectively. In contrast, intense downregulation of inducible Hsp72 expression was confirmed on 10-day obstructed kidney cytosol when compared to the cytosol cortex tissue obstructed for 5 and 7 days. No detectible inducible Hsp72 protein levels were demonstrated following obstruction for 14 days. The behavior of contralateral kidney shows a timing upregulation of inducible Hsp72 protein levels with the highest amount of protein levels after 14 of obstruction, this last one showing a cytoprotective role of the contralateral kidney (Fig. 5).

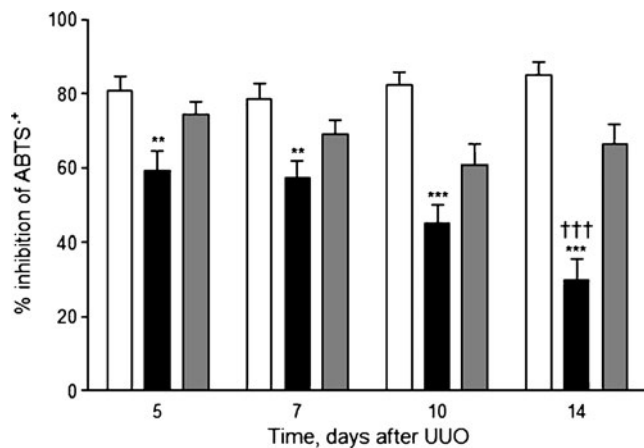
**Table 2** Kidney weight/body weight ratio (KW/BW) [mg/g] from animals after 5, 7, 10, and 14 days of obstruction and from sham-operated rats

Time	Kidney weight/body weight [mg/g]		
	Sham-operated group	Unilateral ureteral obstructive group	
		Control kidney (CK)	Obstructive kidney (OK)
Days of UUO			
5	8.30±0.20	7.49±0.23	6.63±0.22
7	9.12±0.19	7.81±0.31	10.12±0.29
10	6.43±0.25	4.95±0.43 <sup>a,b</sup>	8.22±0.37
14	6.15±0.29	3.75±0.36 <sup>a,b</sup>	7.37±0.33

Values are means±SEM ( $n=6$ )

<sup>a</sup>  $p<0.01$  KW/BW from obstructed kidneys vs. controls

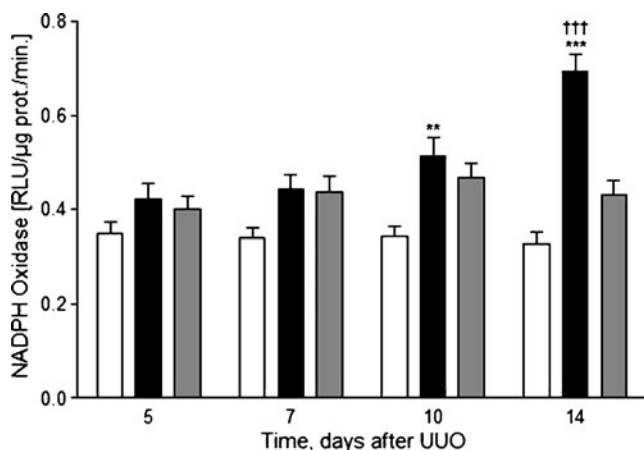
<sup>b</sup>  $p<0.01$  KW/BW from obstructed kidneys vs. contralateral kidneys



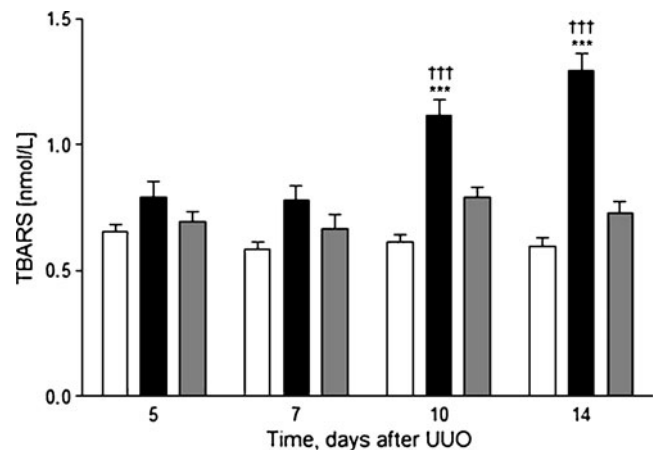
**Fig. 1** Oxidative stress markers at varying time intervals following neonatal UUO. *TTA* total antioxidant activity. Total Antioxidant activity was measured as percent inhibition of  $ABTS^+$ , determined as:  $\% \text{ inhibition} = [(A_0 - A_f)/A_0] \times 100$ . See the “Materials and methods” and “Results” sections for further explanations. Lower antioxidant activity was shown in renal cortex from rats after 5 and 7 days of UUO (*solid bars*) related to controls (*open bars*),  $**p < 0.01$  and intensive decrease on antioxidant activity was demonstrated after 10 and 14 days of obstruction vs. control,  $***p < 0.001$  both; 14-day obstructed kidney vs. contralateral kidney (*shaded bars*),  $†††p < 0.001$ . No differences were shown between control and contralateral cortex. The results shown are representative of four similar experiments

#### Time course of Nrf2 and Keap1 protein levels in progressive obstruction-induced nephrotoxicity

Active Nrf2 accumulates in the nucleus and drives gene expression from antioxidant response elements, restoring



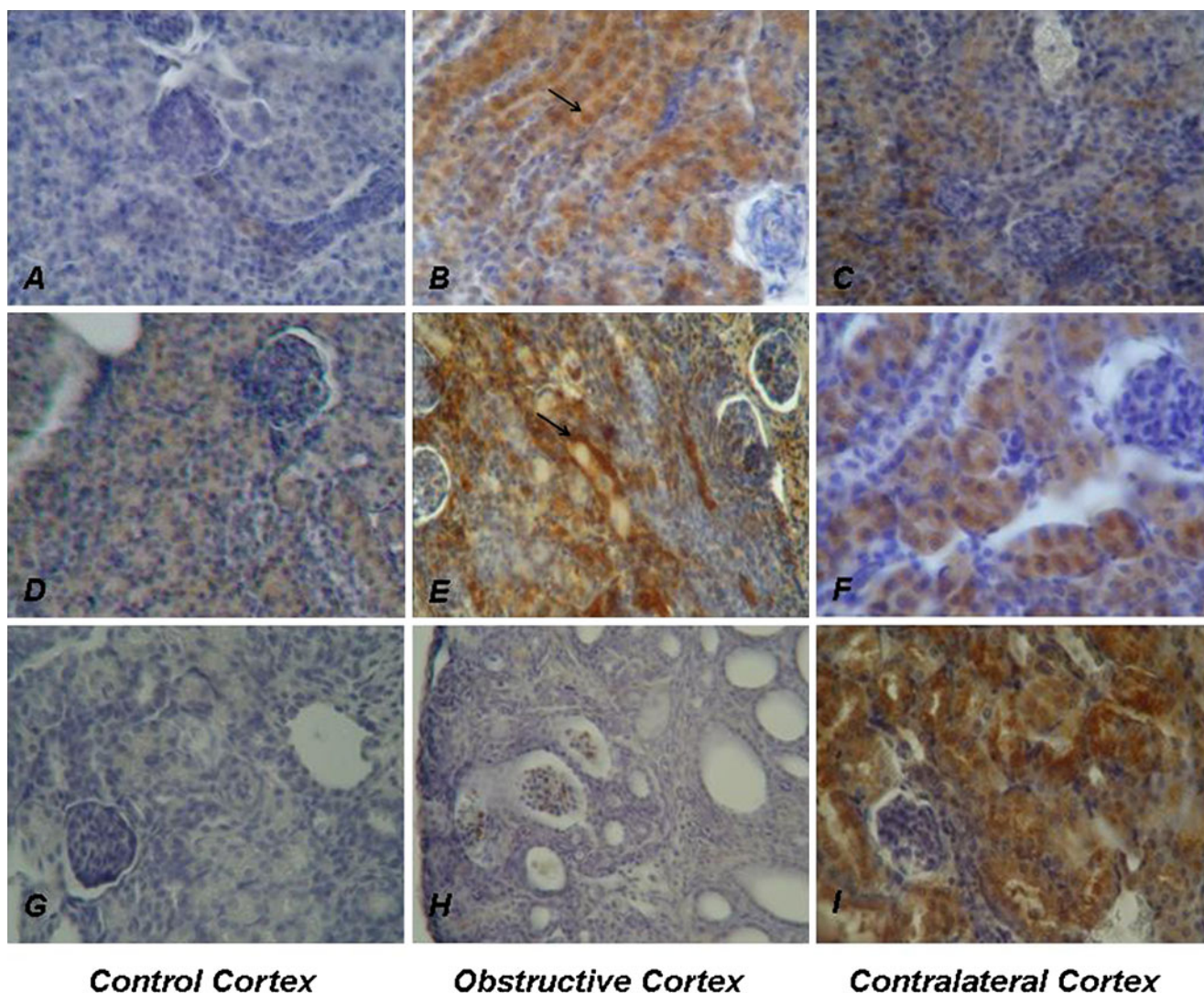
**Fig. 2** Oxidative stress markers at varying time intervals following neonatal UUO. NADPH oxidase activity. Bar graphs depict NADPH oxidase activity in the cortical tissue homogenate of the obstructed (*solid bars*), contralateral (*shaded bars*), and control (*open bars*) kidneys from UUO and control groups. NADPH oxidase activity was measured by a chemiluminescence assay. Increased NADPH oxidase activity was shown after 10 and 14 days of obstruction vs. controls,  $**p < 0.01$  and  $***p < 0.001$ , respectively;  $†††p < 0.001$  obstructed vs. contralateral kidneys. Each *bar* represents the mean  $\pm$  SEM of four separate experiments



**Fig. 3** Lipid peroxidation marker at varying time intervals following neonatal UUO. Thiobarbituric acid reactive substances (TBARS) assay. Bar graphs depict generation of TBARS in the cortical tissue homogenate of the obstructed (*solid bars*), contralateral (*shaded bars*), and control (*open bars*) kidneys from UUO and control groups. Decreased TBARS levels was shown following 10 and 14 days of obstruction vs. controls  $***p < 0.001$  both and  $†††p < 0.001$  obstructed vs. contralateral kidneys, respectively. Data are means  $\pm$  SEM;  $n = 4$  in each group

redox balance. To evaluate Nrf2 expression, tissue cortex was assayed for timing nuclear expression of Nrf2 by western blot. Unilateral obstruction for 7 days induced a robust nuclear accumulation of Nrf2 whereas decreased Nrf2 protein levels were shown in cytosol fraction. In contrast, intense decrease on nuclear Nrf2 following obstruction for 14 days was demonstrated, with cytosol Nrf2 protein abundance recovery, indicating absence of an antioxidant response at this time course (Fig. 6).

Nrf2 is sequestered in the cytoplasm, where it is associated with Keap1 under unstimulated conditions (Hayes and McMahon 2001). Nrf2 can be translocated into the nucleus to bind to the ARE with small Maf proteins when the Keap1–Nrf2 complex is disrupted under some cellular stimuli (Itoh et al. 1999). Thus, obstruction-induced Nrf2–ARE activation may be linked to the relative level of Nrf2–Keap1. Enhanced Nrf2 protein level reduces Keap1 protein level. For this, we examined the prolonged effects of obstruction on the endogenous cytosolic Nrf2–Keap1 expression. As shown in (Fig. 6), obstruction for 7 days enhanced the level of endogenous nuclear Nrf2. In contrast, reduction of cytosol Keap1 protein levels was shown during the same period of obstruction. These results suggest that early obstruction (7 days) may stimulate Nrf2-mediated ARE activation by enhancing Nrf2 nuclear protein level and reducing cytosol Keap1 at the same time, in other words, by increasing the ratio of Nrf2/Keap1 (1.36). In contrast nuclear Nrf2 protein expression was mildly reduced at 10 days of obstruction. Intensive decrease of nuclear Nrf2 protein expression after 14 days of obstruction



**Fig. 4** Histologic sections of neonatal kidney cortex following unilateral obstruction for 5, 7, and 14 days. Localization of Hsp70 expression after 5 days of obstruction in control (**a**), obstructed (**b**), and contralateral histologic cortex kidney sections (**c**). Hsp70 expression in cytoplasm and in tubule epithelial cells from cortical collecting ducts and proximal tubules. Slight tubular epithelial cells Hsp70 immunoreaction was shown in control (**d**) and contralateral

cortex (**f**) after 7 days of obstruction, strong Hsp70 immunoreaction in CCD and PT epithelial cell cytoplasm and membrane in obstructed cortex (**e**). After 14 days of obstruction weak Hsp70 immunoreaction in the cytoplasm of the same epithelial cells was shown (**h**). In contrast, higher immunostaining for Hsp70 in contralateral kidney cortex section was shown (**i**). Magnification 400 $\times$

was associated with increased cytosolic Keap1 protein abundance and decreased Nrf2/Keap1 ratio (0.21).

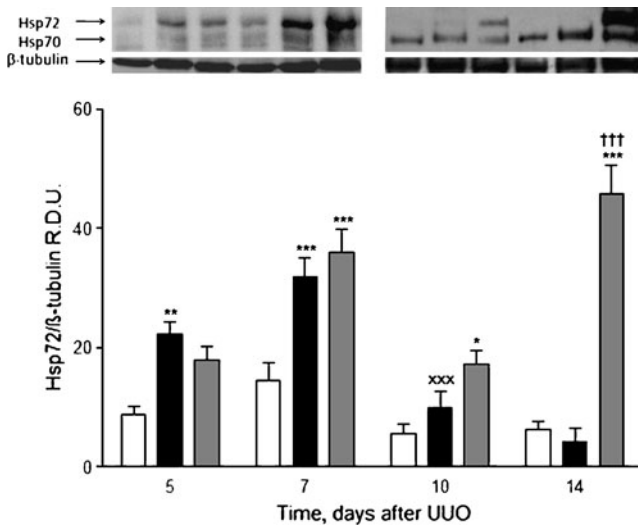
The presence of Nrf2 in the nucleus turn on the expression of the ARE genes

An important mechanism by which cells adapt to oxidant stress is to transcriptionally upregulate a distinct array of cytoprotective genes responsible for buffering the cells' antioxidant capacity. To confirm the presence of the antioxidant response, the expression of the Nrf2 target genes GSTA2 and NQO1 were determined in obstructed kidney cortex. Higher mRNA expression of GSTA2 and

NQO1 were demonstrated after 7 days of obstruction by twofold increase of control both, respectively. Prolonged obstruction showed nuclear Nrf2 content reduction, signifying diminished activation of these transcription factors. On this way, near twofold decrease on NQO1 and GSTA2 mRNA was shown in 14-day obstructed kidneys compared to 7-day obstructed kidneys (Fig. 7).

## Discussion

Congenital obstructive nephropathy reveals that obstruction to urine flow in the developing kidney either impairs

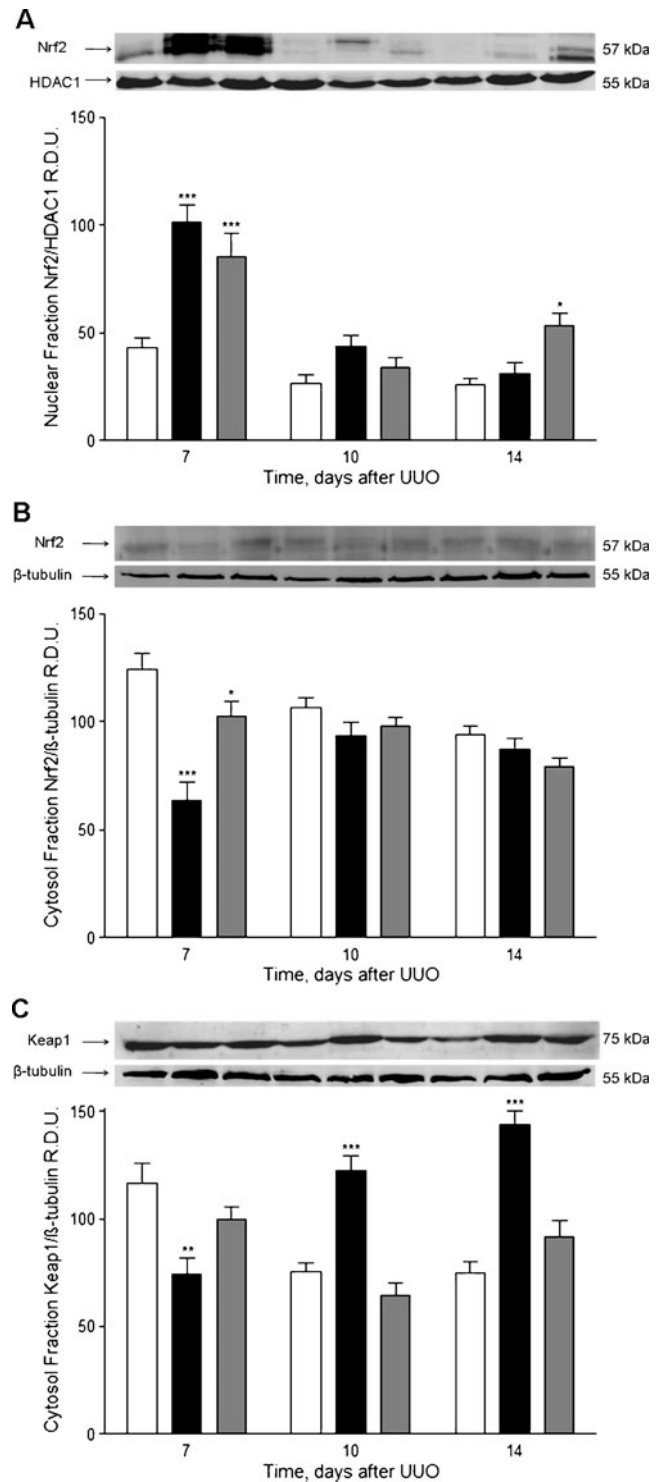


**Fig. 5** Cytoprotection afforded by the heat shock response in early neonatal unilateral ureteral obstruction (UUO). Cytosol preparations from renal cortex were used for western blot analysis. Antibody against Hsp70 protein recognized a band at 70 and 72 kDa (inducible). As loading controls, cytosolic extracts were probed for  $\beta$ -tubulin. Densitometric analysis revealed higher abundance of the inducible form Hsp72 in cytosol cortex fraction from 5 to 7 days obstructed kidneys vs. control,  $**p < 0.01$  and  $***p < 0.001$ , respectively. In contrast, intensive decrease of inducible Hsp72 was shown in obstructed cytosol cortex fraction for 10 days when compared to 5 and 7 days obstructed cortexes,  $xxxp < 0.001$  both. Absence of inducible form Hsp72 was demonstrated in 14-day obstructed cortex. Progressive increase of inducible Hsp72 in contralateral kidney was shown. After 14 days of obstruction, increased expression of Hsp72 was demonstrated in contralateral vs. obstructed kidney,  $†††p < 0.001$ . Data represent the mean  $\pm$  SEM;  $n = 4$ . RDU relative densitometric units

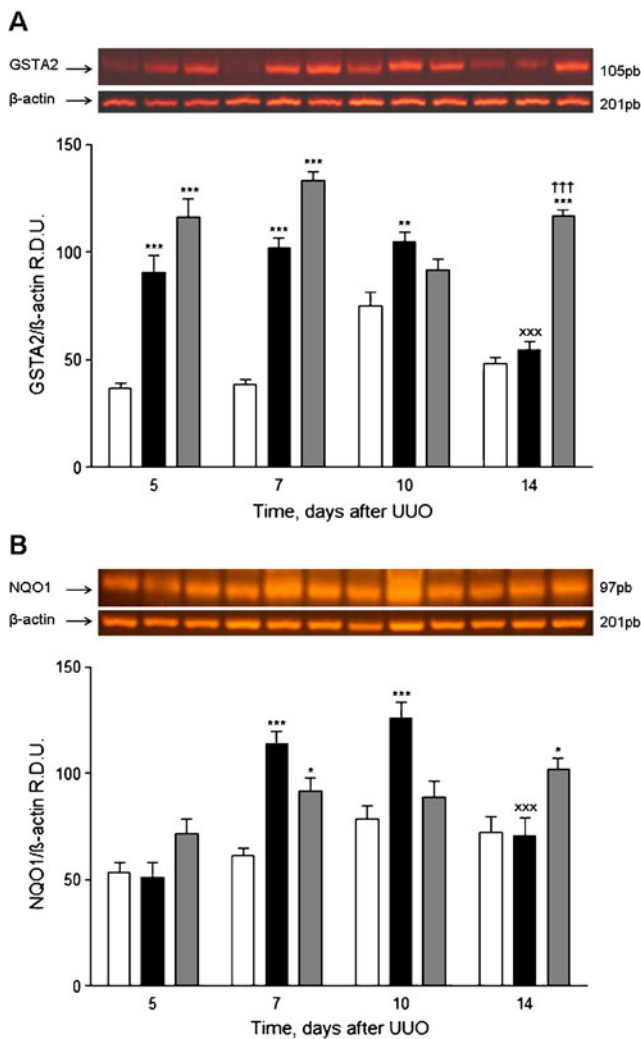
nephrogenesis or leads to nephron loss (Chevalier 1999). Mechanical stretch of the tubular epithelium and oxidative stress are believed to be early stress factors leading to tubular cell injury and death. Following an initial increase

**Fig. 6** Time course of the Nrf2–Keap1 cellular defense pathway in neonatal unilateral obstruction. Cortex homogenates from obstructed, contralateral, and control kidneys were isolated and fractionated into nuclear and cytosol extracts (see the “Materials and methods” section). Equal amounts of protein extracts were loaded in each lane, resolved by SDS-polyacrylamide gel electrophoresis, and probed for Nrf2 (nuclear and cytosol extracts) and Keap1 (cytosol extract) by immunoblot analysis. As loading controls, cytosol extracts were probed for  $\beta$ -tubulin and nuclear fractions were probed for HDAC1. Densitometric analysis revealed Nrf2 higher abundance in nuclear cortex fraction (a) from 7-day obstructed kidney vs. control,  $***p < 0.001$  and contralateral nuclear fraction vs. control,  $***p < 0.001$ . In contrast, in cytosol cortex fraction (b), decreased Nrf2 expression was shown in 7-day obstructed kidney vs. control,  $***p < 0.001$ . Prolonged obstruction for 14 days blunted nuclear translocation of Nrf2 whereas increased cytosol Nrf2 protein level was shown. Keap1 is a negative regulator of Nrf2. Lower cytosol Keap1 (c) protein abundance was shown in 7-day obstructed kidneys vs. control,  $**p < 0.01$ . Conversely, increased Keap1 expression was shown in cytosol fractions (c) from 10- to 14-day obstructed kidneys vs. control,  $***p < 0.001$  both. Data represent the mean  $\pm$  SEM;  $n = 4$

in blood flow to the kidney, renal blood flow decreases markedly in chronic obstruction and ischemia is likely to also contribute to tubular cell injury (Gulmi et al. 2002; Klahr and Morrissey 2002). The degree of oxidative stress and the severity of subsequent renal fibrosis may depend on an imbalance between excessive production of ROS and







**Fig. 7** Activation of the antioxidant transcription factor Nrf2 mediates cytoprotective gene expression in early neonatal unilateral obstruction. Representative gels of NQO1 (a) and GSTA2 (b) mRNA from obstructed, contralateral, and control cortex kidneys after 5, 7, 10, and 14 days of obstruction, are shown. The corresponding housekeeping  $\beta$ -actin is included in below. Histograms show the relative concentration of NQO1 and GSTA2 mRNAs to  $\beta$ -actin mRNA. \*\*\* $p < 0.001$  vs. control \*\* $p < 0.01$  vs. control ††† $p < 0.001$  14-day obstructed kidney vs. contralateral kidney. xxx $p < 0.001$  14-day obstructed vs. 7-day obstructed kidney. Data represent the means  $\pm$  SE of four independent experiments

antioxidant defense within the kidney (Sunami et al. 2004; Lee and Johnson 2004). In this study, the time course tubular cell adaptation to oxidant stress in obstruction was studied through the activation of antioxidant cytoprotective genes and heat shock protein response pathways. Our data reveal an early Hsp70 response and Nrf2 nuclear activation, the latter driving dependent cytoprotective gene expression, counteracting the oxidative stress effects to kidney obstruction for 7 days. In contrast, the significant reduction on the Hsp70 inducible form expression and lack of antioxidant responsive elements activation because of Nrf2 down-

regulation and increased Keap1 expression, directly correlate with higher oxidative stress markers after renal obstruction for 14 days.

Intensive intrarenal oxidant stress in obstruction has been described, due to either a direct effect of macrophages or tubular-derived ROS production (Ricardo and Diamond 1998). Oxidative stress is caused by a combination of increased production of reactive oxygen species ROS (Vaziri 2004; Manucha et al. 2005) and impaired antioxidant capacity (Ishii et al. 2002; Jong-Min and Johnson 2004). Increased ROS may be critical; playing a role in inducing an inflammatory state and in deteriorating the renal function by activating redox-sensitive transcription factors and signal transduction pathways. These events, in turn, promote necrosis, apoptosis, inflammation, and fibrosis (Haugen and Nath 1999). Previously, in kidney slices obtained from UUO kidneys, higher superoxide anion and hydrogen peroxide and a corresponding decrease in catalase and copper–zinc superoxide dismutase mRNA release have been demonstrated (Ricardo and Diamond 1998). Increased 8-hydroxy-2'-deoxyguanosine and heme oxygenase-1 (HO-1) as markers of oxidative stress in UUO (Kawada et al. 1999; Pat et al. 2005) and upregulation of HO-1 that provided protection against renal injury in UUO (Kim et al. 2006) have been reported. In this study, we have demonstrated lipid peroxidation through higher TBARS levels in the obstructed kidney, and increased oxidative stress in a UUO model, resulted in reduced TAA and enhanced NADPH oxidase activity in 14-day obstructed cortex. In contrast, after 5 and 7 days of obstruction, a slight reduction in TAA without increase on renal cortex NADPH oxidase activity (a major source for superoxide production) and absence of increase on lipid peroxidation products were shown. The increased vascular resistance in obstruction contributes to renal ischemia and hypoxia, both factors that have been demonstrated to be involved in the induction of stress protein Hsp70 (Van Why et al. 1992). Our data reveal a role for the heat shock response in counteracting the oxidative stress induction after 5 and 7 days of unilateral obstruction. Hence, activation of the Hsp70 response significantly attenuated the degree. Increased lipid peroxidation and NADPH oxidase activity associated with intensive Hsp70 downregulation confirmed by immunohistochemical and western blot studies. In response to unilateral ureteral obstruction, the contralateral kidney undergoes compensatory renal growth which is enhanced in early development. The mechanisms responsible for compensatory renal growth remain incompletely understood. A number of studies point to a combination of release of renotropic factors and suppression of inhibitors of renal growth (Yoo et al. 2006). After 14 days of obstruction, in intact opposite kidney upregulation of inducible Hsp70 protein expression occurred in parallel to protection against oxidative stress. The

consequences of heat shock response activation by Hsp70 induction likely extend beyond cytoprotection from oxidative stress. Hsp70 has been shown to directly inhibit a variety of signaling pathways, including the lipopolysaccharide-induced innate immune response. As a consequence, Hsp70 attenuates the induction of proinflammatory genes, including cyclooxygenase-2, inducible nitric oxide synthase (NOS), tumor necrosis factor  $\beta$ , interleukin-1 (IL-1), and interleukin-6 (IL-6; Lau et al. 2000; Manucha and Vallés 2008; Chen et al. 2006).

In view of the complex biology of oxidative stress, in our experiments, the magnitude of cytoprotection exceeded the contribution of Hsp70. Accordingly, Nrf2 and induction of the antioxidant response were demonstrated to be undoubtedly significant on 7-day obstructed kidneys (early renal obstruction). The ARE is a *cis*-acting regulatory element or enhancer sequence, which is found in promoter regions of genes encoding phase II detoxification enzymes and antioxidant proteins such as NQO1 (Li and Jaiswal 1992) and GSTA2 (Rushmore 1990). Nrf2 regulates basal activity and coordinated induction of genes encoding antioxidant and phase II detoxifying enzymes including detoxification enzymes (GST and NQO1), and antioxidant enzymes (catalase, glutathione peroxidase, superoxide dismutase, and HO-1) (Li et al. 2008). Via activation of Nrf2 and consequent expression of the antioxidant and detoxifying enzymes, ROS elicit a compensatory response aimed at mitigating the impact of oxidative stress (Jaiswal 2004). In fact, there is growing evidence supporting the protective role of Nrf2-mediated pathway against oxidative stress and inflammation. It has been widely demonstrated that Nrf2 activation occurs due to an alteration in the redox state of the cell due to the presence of increased amounts of electrophiles or ROS. In this context in a recent work, ROS generation in a model of hypoxia/reoxygenation of renal epithelial cells have led to the Nrf2 activation, an antioxidant response to protect them from future oxidant damage (Leonard et al. 2006). In our study, in spite of severe oxidative stress which should have induced activation of Nrf2 and upregulation of its downstream gene products, prolonged obstruction showed progressive reduction of nuclear Nrf2 content. The diminished activation of this transcription factor after 14 days of renal obstruction was accompanied by a significant downregulation of the Nrf2 target gene products including the detoxifying enzymes NQO1 and GSTA2. By contrast, on early obstruction for 7 days, we have demonstrated decreased activity of oxidative markers associated with a rapid nuclear accumulation of Nrf2 and induction of promoter reporter activity of two of the identified Nrf2-dependent genes NQO1 and GSTA2. The above events draw attention to the impaired ability of time-persistent obstruction to mount the biological response to the prevailing oxidative stress leading to renal injury.

Detailed analysis of the regulatory mechanism governing Nrf2 activity led to the identification of Keap1, which represses Nrf2 activity by directly binding the N-terminal Neh2 (Itoh et al. 1999). Keap1 interaction with Neh2 domain of Nrf2, conducts to the sequestration of Nrf2 in the cytoplasm and to the enhancement of Nrf2 degradation by proteasomes conferring tight regulation on the response (McMahon et al. 2003; Stewart et al. 2003). Constitutive activation of Nrf2-regulated transcription in Keap1 knockout mice clearly demonstrated that the disruption of Keap1 repression is sufficient for the activation of Nrf2 (Wakabayashi et al. 2003). Confirming this role, in the presence of severe oxidative stress, a significant increase of Keap1 protein level in 14-day obstructed kidney was associated with unexpected reduction of Nrf2 activation and of its target gene products. In contrast in early 7-day obstruction, absence of a positive regulation of Keap1 protein dissociating the Nrf2–Keap1 complex, allowed to Nrf2 translocation into the nucleus where it transcriptionally activated downstream target genes. Owing to Keap1 regulation of both cytoplasmic-nuclear shuttling and degradation of Nrf2, the absence of Nrf2 activation despite the prevalent oxidative stress in 14 day obstruction demonstrated here, could be explain, in part, through the increased Keap1 protein levels. Previously, in an experimental model of chronic renal failure, despite severe oxidative stress and inflammation; remnant kidney tissue Nrf2 activity (nuclear translocation) was markedly reduced, whereas the Nrf2 repressor Keap1 was upregulated and the products of Nrf2 target genes were significantly diminished (Kim and Vaziri 2010). A dissociated behavior of the intact opposite kidney through the increased Hsp70 protein expression that occurred in parallel to the activation of Nrf2-antioxidant response element signaling pathway, allow us to suggest a protective role of the contralateral kidney after 14 days of unilateral obstruction.

This study represents a longitudinal investigation of the renal cytoprotective cellular response through Hsp70 upregulation and specific activation of Nrf2 that results in accumulation and transactivation of Nrf2-dependent antioxidant gene expression, being a vital mechanism through which these genes are upregulated in early obstruction to protect the cell from further damage. Time-prolonged obstruction for 14 days led to acquired deficiency of the Nrf2 pathway contributing to the progressive oxidative stress and the severity of renal injury.

In summary, we have demonstrated for the first time the induction of Nrf2 activation and Nrf2-dependent ARE-driven antioxidant gene expression in an *in vivo* model of neonatal UUO associated with increased Hsp70 expression counteracting the oxidative insult, in early obstruction (7 days). We also demonstrate lack of cytoprotective Hsp70 response and impaired Nrf2–Keap1 system in

the regulation of cellular defense against prolonged obstruction-induced oxidative stress by upregulation of Keap1 and reduced Nrf2-dependent transcriptional downstream target genes activation transcriptionally activates downstream target genes.

As stated for our results we speculate the magnitude of cytoprotection in early obstruction depends on the combined contribution of induced activation of Nrf2 upregulating its downstream gene products and heat shock response.

Further investigation into the signaling pathways involved in this response may help our understanding of obstruction-induced oxidative stress toward future therapeutic potential.

**Acknowledgments** This work was performed with financial from CONICET, PICT/2005N°33827 and from the Research and Technology Council of Cuyo University (CIUNC) Mendoza, Argentina/N: 658/05 to P.G. Vallés.

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