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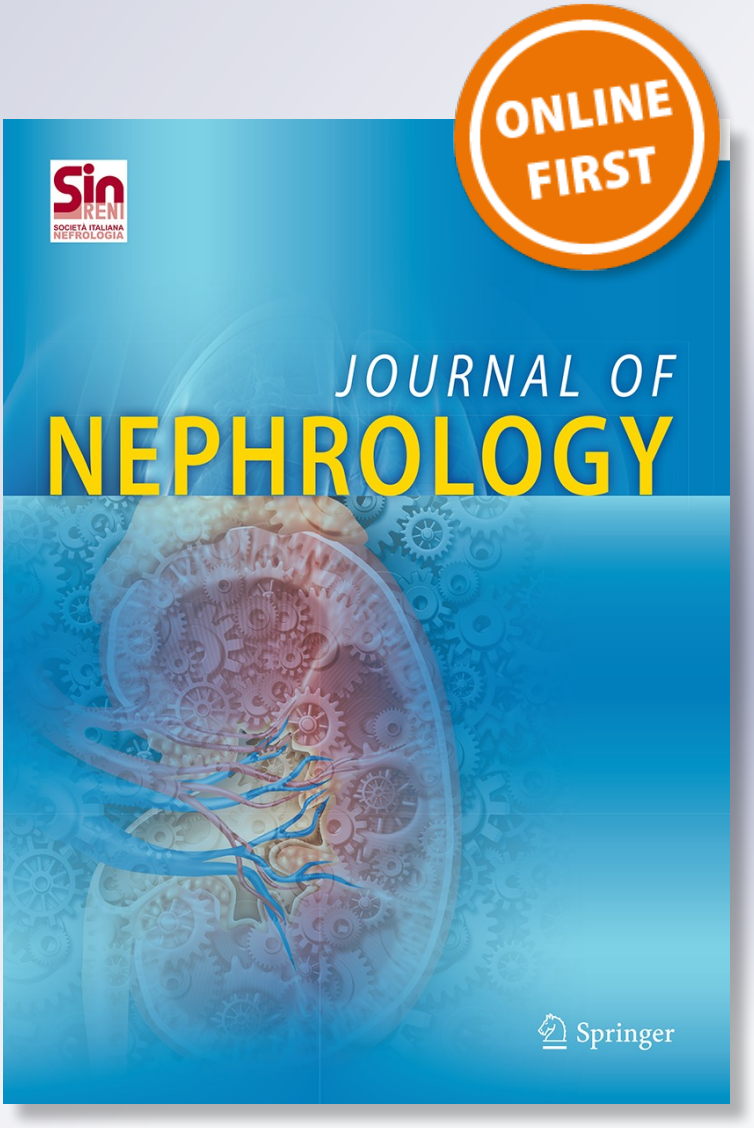

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REVIEW

Growing evidence suggests WT1 effects in the kidney development are modulated by Hsp70/NO interaction

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Abstract The study of kidney development at the cellular and molecular levels remains an active area of nephrology research. The functional integrity of the kidney depends on normal development as well as on physiological cell turnover. Apoptosis induction is essential for these mechanisms. A route to cell death revealed in the past decade shows that heat shock proteins (HSPs) and their cofactors are responsible for regulating the apoptotic pathway. Specifically, heat shock protein 70 (Hsp70), the most ubiquitous and highly conserved HSP, helps proteins adopt native conformation or regain function after misfolding. Hsp70 is an important cofactor for the function of Wilms' tumour 1 (WT1) and suggests a potential role for this chaperone during kidney differentiation. In addition, we have demonstrated that WT1 expression is modulated by nitric oxide (NO) availability and Hsp70 interaction after neonatal unilateral ureteral obstruction. NO has been identified as playing an important role in the developing kidney. These findings suggest that Hsp70 and NO may play a critical and fundamental role in the capacity to modulate both apoptotic pathway and oxidative stress during kidney development. Furthermore, the design of experimental protocols that assess renal epithelial functionality in this context, could contribute to the understanding of renal development and alterations.

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Keywords Kidney development · Wilms' tumour 1 · Heat shock protein 70 · Nitric oxide · Apoptosis

Introduction

The functional integrity of the kidney depends on normal development as well as on physiological cell turnover. Apoptosis induction is essential for these mechanisms to occur. Hence, when cells are stressed, a common response is to undergo cell death. A route to cell death revealed in the past decade shows that heat shock proteins (HSPs) and their cofactors are responsible for regulating the apoptotic pathway [1].

The induction of stress response (heat shock) proteins (HSPs) is a conserved mechanism that protects many cell types from diverse physiological and environmental stressors. HSP families of different sizes function as molecular chaperones that modulate the folding of proteins into functional conformations. HSPs are believed to facilitate the restoration of normal function by assisting in the refolding of denatured proteins and in the degradation of irreparably damaged proteins [2]. Specifically, heat shock protein 70 (Hsp70), the most ubiquitous and highly conserved HSP, helps proteins adopt native conformation or regain function after misfolding [3]. The Hsp70 family consists of molecular chaperones of approximately 70 kDa in size that serve critical roles in protein homeostasis. They also protect nascently translating proteins, promote the cellular or organellar transport of proteins, reduce proteotoxic protein aggregates, and serve general housekeeping roles in maintaining protein homeostasis [4]. Finally, and of great significance, the study of kidney development at the cellular and molecular levels remains an active area of Wilms' tumour 1 (WT1). Interestingly, the WT1 gene

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plays an important role at three different stages of kidney development: the onset of kidney formation, the progression of kidney formation, and the maintenance of normal kidney function [5]. And, particularly relevant to our area of knowledge, Hsp70 is an important cofactor for the function of WT1 and suggests a potential role for this chaperone during kidney differentiation [6] (Fig. 1). Moreover, we have proposed that WT1 mRNA expression is modulated by nitric oxide (NO) availability and Hsp70 interaction after neonatal unilateral ureteral obstruction (UUO) [7] (Table 1). A previous work has demonstrated a functional WT1/inducible nitric oxide synthase (iNOS) promoter interaction [8] (Fig. 1). NO, a simple molecule synthesized from L-arginine by NO synthases, has been identified as playing an important role in regulating adult renal function, and original research points to perhaps an even more critical role for NO in the developing kidney [9]. In addition, our work in neonatal obstructive nephropathy has shown low p53 and Hsp70 expressions, which are increased in association with higher NO levels under pharmacologic treatment. Conversely, Madin–Darby canine kidney (MDCK) cells with NO deprivation have expressed low Hsp70 and p53 mRNA levels. These observations suggest a potential role for NO bioavailability and Hsp70 interaction during kidney differentiation [10] (Table 1).

WT1 in kidney development

Normal kidney development is critical for survival; the molecular mechanisms underlying the development of this organ, however, are only beginning to be elucidated. The kidney as a regulator of the internal environment is an extremely complex structure that deals with important endocrine functions including, among others: the production of renin, prostaglandins and erythropoietin; metabolic waste removal; control and regulation of acid–base status,

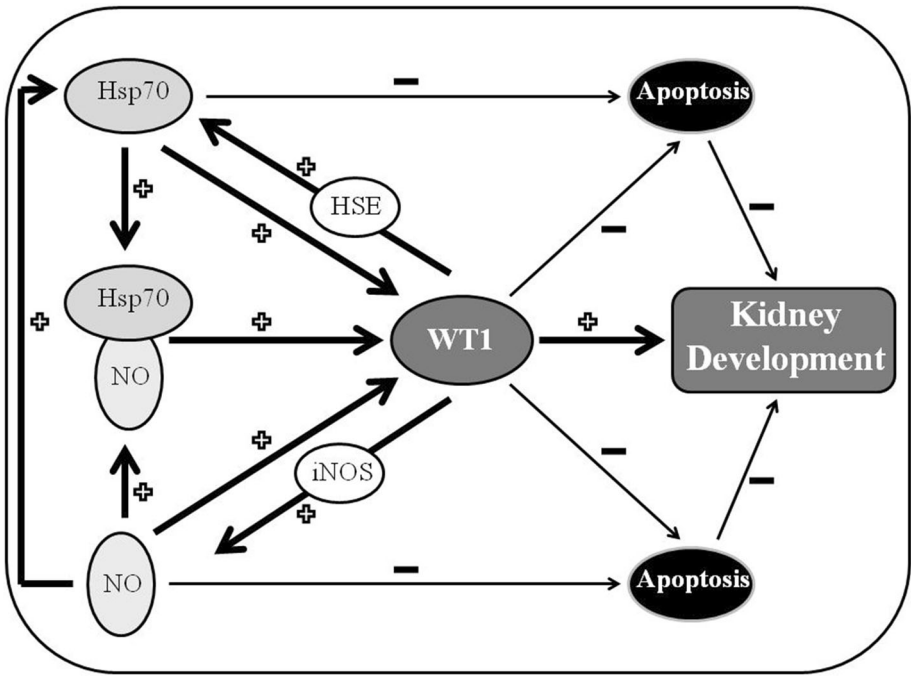

Fig. 1 Graphical overview: the potential for interaction between Hsp70 and NO in relation to kidney nephrogenesis could be a consequence of WT1 modulation

Table 1 Pharmacological studies on UUO with regard to WT1, Hsp70 and NOS modulation

Drugs	Evaluated factors	References
Statin and nitric oxide modulators (original article)	WT1/NO/Hsp70	Mazzei et al. [7]
Statin and nitric oxide modulators (review)	WT1/NO/Hsp70	Mazzei and Manucha [10]
Statin (original article)	WT1	Mazzei et al. [26]
Losartan (original article)	Hsp70	Manucha et al. [42]
Statin (original article)	NO/Hsp70	Manucha et al. [55]

UUO unilateral ureteral obstruction, *WT1* Wilms' tumour 1, *Hsp70* heat shock protein 70, *NO* nitric oxide, *NOS* nitric oxide synthase

and volume and composition of extracellular fluid. Three sets of kidneys develop during embryogenesis, i.e. the pronephros, the mesonephros and the metanephros [11]. The nephron, the renal functional unit, is constituted by about 10,000 cells, and these, together with the collecting ducts, form the uriniferous tubules. At least 14 different cell subtypes, perfectly organized and placed into different segments of the tubules, have been characterized. This implies that the renal morphogenesis should be perfectly regulated during development, so that each particular cell type is placed in its corresponding end location, relative to other cell types along the tubules. The precursors of the permanent kidney in the metanephros result from reciprocal interactions between the epithelial ureteric bud and the pluripotent metanephric mesenchyme. The mesenchyme induces the bud to grow and branch, thus forming the ureter, renal pelvis and collecting ducts. The ureteric bud induces the mesenchyme to undergo an epithelial transformation, to condense and form the mature nephron with the glomerulus, proximal convoluted tubule, loop of Henle and distal convoluted tubule [12]. Therefore, renal development requires the conversion of mesodermal cells into polarized epithelial mesenchymal cells [13], which is one of the processes taking place during the kidney, gonads and mesothelium organogenesis. The number of mature cells in a tissue depends on the balance between mitosis and apoptosis. In the kidney, the cells remaining around the mesenchyme show morphological and biochemical features of programmed cell death. This is consistent with recent ultrastructural observations of apoptosis during metanephric development [14].

In the past decade, several genes have been identified that exhibit spatially and temporally distinct expression patterns during kidney development and lead to abnormal organ development when disrupted by gene targeting experiments [15]. Particularly noteworthy, WT1 has been identified as a gene that controls transitions between the mesenchymal-to-epithelial states of cells. It is absent or mutated in embryonic kidney cancer cells. This gene is located on chromosome 11 (11p13) and encodes a protein rich in proline and glutamine in the amino-terminus, and four (cis)2-(his)2 zinc fingers in the carboxy-terminus [16]. There is evidence that WT1 may be directly involved in the regulation of proliferation and cell differentiation. In situ hybridization studies have shown that WT1 is selectively expressed in the metanephric blastema and glomerular epithelium during embryonic and fetal development [17]. The expression patterns of WT1 [18] indicate an important role of this gene not only during urogenital development, but also during fetal and postnatal life. In addition, using laser microscopy techniques, WT1 was sub-located in the nucleus, regardless of cell type and stage of development. Moreover, it has been concluded that the initial growth of the ureteric bud is dependent on a signal from the metanephric blastema and WT1 would be essential for this process [19–21]. Also, previous reports have established the effect of a mutation in WT1 and that its deficiency affects the expression of Pax-2 (a regulatory factor present during kidney development) [22], which modulates the Wolffian duct, the ureteric bud and the metanephric mesenchyme. E11 embryo mutated sections and "wild type" sections for WT1 were obtained and incubated with a Pax-2 polyclonal antibody: a signal was obtained in the Wolffian duct in both the mutant and the "wild type" sections; however, no expression was detected in the mutant metanephric blastema, which indicates the necessity of WT1 for the expression of Pax-2 [23].

It should be noted that the physiologic developmental kidney program is disturbed in the most advanced cases of congenital obstructive nephropathy, arguing for altered temporal/spatial expression of genes which control normal nephrogenesis [24]. Thus, obstructive nephropathy in neonatal rodents can be used as a paradigm for studying affected kidney development. In addition, major regulators of mesenchymal–epithelial transformation and collecting duct and tubular development such as WT1 and Sall1 are decreased with obstruction [24]. Indeed, we demonstrated WT1 down-regulation during UUO [7, 10, 25, 26]. Apoptosis is, consequently, an unquestionable process mediated by the decreased expression of apoptosis genes, as well as by the over-expression of pro-apoptotic genes [25]. Also, as regards the preservation of renal structure, we have shown that statin treatment resulted in the maintenance of gene expression rates for genes that are key markers of renal development [26] (Table 1). WT1, a master regulator of nephrogenesis, has been shown to be maintained by the rosuvastatin treatment. Moreover, the E-cadherin gene is a key target of WT1 transactivation during mesenchymal-to-epithelial transition (MET) [27] and the preservation of E-cadherin levels in the obstructed kidneys from rosuvastatin treated animals suggests that the epithelial structure is being maintained in terms of fundamental cell adhesion and polarity. The normalization of BMP-7 expression indicates that another key inductive signal for MET events, crucial to the later stages of nephrogenesis, is sustained by statin [28]. WT1 transactivation requires phosphorylation. WT1 functions as a transcriptional regulator and its activity is controlled through phosphorylation by protein kinase A (PKA). PKA-dependent WT1 phosphorylation results in translocation of WT1 from the nucleus to the cytosol, a process that interferes with WT1 transcriptional activities [29]. In addition, the WT1 gene is characterized by a plethora of different isoforms with, in some cases, clearly different functions in transcriptional control and RNA metabolism [30]. Furthermore, a recent finding suggests important roles for microRNAs (miRNAs) in general—and

for specific miRNAs in particular—in normal kidney development that still await further analysis [31].

The identification of miRNA processor mutations in Wilms' tumors emphasizes the importance of miRNAs in normal kidney development, in particular in the nephron progenitor cells. This pathway produces mature miRNAs that negatively regulate protein expression by targeting mRNAs. Indeed, disruption of the miRNA processing pathway in Wilms' tumor arose after identification of inactivating DIS3L2 [32]. DIS3L2 is involved in recognition and degradation of polyuridylated mRNA and miRNAs. Specific targets of DIS3L2 include uridylated pre-let-7 miRNAs, performed by LIN28-activated TUT4 and TUT7 enzymes. By binding to the Let-7 premiRNA loop, LIN28 blocks DICER processing and therefore expression of mature let-7 miRNAs in undifferentiated cells during development, maintaining cell renewal and pluripotency (for a review see [31]). Also, Six2-Cre-mediated knockout of Dicer1 has already demonstrated the importance of miRNAs in the nephron progenitor cells [33], and specific miRNAs found deregulated in Wilms' tumors would be strong candidates to analyze for their roles. In addition, several studies have identified miRNAs, such as miR-23a, that are down-regulated in Wilms' tumor and impact on expression of key genes involved in renal development. Thus, HOXB4 overexpression results in persistent embryonic renal [34]. Therefore, by failing to generate mature miRNAs, the normal nephrogenic differentiation process cannot continue, as primordial renal genes cannot be silenced, and cells remain stuck in a precursor-like state.

Hsp70 and kidney development

The expression of HSP is tightly regulated during development in multiple organs. As housekeeping proteins, small heat shock proteins help to protect cells from apoptosis, stabilize the cytoskeleton and contribute to proteostasis. During renal development the cells in the medulla are exposed to elevated and variable interstitial osmolality. Hsp70 is a major molecular chaperone and plays an important role in the protection of cells in the renal medulla from high osmolality [35]. Hsp70 displays weak ATPase activity and cyclically binds and releases hydrophobic segments of unfolded and partially folded proteins in an ATP/ADP-dependent reaction cycle. The complex consisting of ADP, Hsp70 and nonnative polypeptides is relatively stable, thus preventing incorrect interaction between protein domains. The exchange of ADP against ATP results in a low-affinity complex that releases the substrate polypeptide rapidly and thus allows the folding process to advance [36–38]. The binding and release of substrate polypeptides to Hsp70 is modulated by cofactors (Hsp40, Hip, BAG) that may also regulate ADP/ATP exchange or ATP hydrolysis [39]. Hsp73 (70-kDa heat shock cognate protein) is the major constitutively expressed member of the Hsp70 family. Hsp73 has been observed in all zones of normal rat kidneys (in the cortex of all tubular epithelial cells, in glomerular podocytes and, though not consistently, in Bowman's capsule). The immunoreactivity for Hsp73 is similar in the nucleus and cytoplasm in most of these cells except for podocytes, Bowman's epithelium, and proximal tubule cells, where nuclear expression is more pronounced. This ubiquitous presence of Hsp73 can be attributed to the need, also of nonstressed cells, for assistance in protein folding, trafficking and controlled degradation. In contrast, in the normal rat kidney, Hsp72 mRNA abundance and the tissue content of Hsp72, the most readily inducible member of the Hsp70 family, increase steeply along the corticopapillary axis. Hsp72 displays a distribution pattern closely paralleling the tissue solute concentration characteristic of the concentrating kidney. As a consequence, Hsp72 was detected in the cortex in individual collecting duct cells only. In the outer medulla, all tubules were stained weakly, whereas in the papilla intense staining of collecting ducts and the epithelium lining the papilla was noted [40]. The intrarenal distribution of Hsp72 and the effect of altered extracellular tonicity on Hsp72 have led to the hypothesis that this HSP assists in the adaptation of medullary cells to high extracellular solute concentrations.

Unilateral ureteropelvic junction obstruction (UPJO) is a condition involving abnormal nephrogenesis with injury to the kidney, leading to vasoconstriction, macrophage infiltration, oxidative stress, and tubulointerstitial fibrosis and apoptosis. In this regard, we analyzed the impact of UPJO on the expression of Hsp70 in 22 children and related the changes to renal function and duration of obstruction. Kidney function was evaluated, and renal biopsies for immunohistochemical and western blot analyses were obtained at the time of surgery. Increased Hsp70, with an expression pattern related to the duration of the obstruction, was noted in proximal tubules (PTs), cortical collecting ducts (CCDs), and medullary collecting ducts. Strong Hsp70 staining in cytoplasm and nuclei of these segments appeared in children with kidney obstruction for 2 years. These results support the concept that Hsp70 is involved in the adaptive response of the human kidney to congenital UPJO [41]. As shown in numerous investigations, Hsp70 may provide cytoprotection against diverse factors; thus, protection against tubulointerstitial fibrosis with Losartan, independent from changes in blood pressure, includes decreased oxidative stress linked to upregulation of Hsp70 expression [42] (Table 1). As previously mentioned, the cellular stress response can mediate cellular protection through the expression of Hsp70, which can interfere with

the process of apoptotic cell death. Accordingly, Hsp70 expression is associated with inhibition of renal tubule epithelial cell apoptosis during recovery from low-protein feeding [43]. Also, translocation of Hsp70 to proximal tubule membranes in Losartan treated spontaneously hypertensive rats (SHRs) might exert a cytoprotective effect by the down-regulation of NADPH subunits Nox4 [44]. Moreover, our data suggest that Hsp70/AT_1 modulated by vitamin D receptor (VDR) is involved in the mechanism by which paricalcitol (a VDR inducer) provides renal protection in SHRs. We propose that low AT_1 expression through VDR induction could be a consequence of the heat shock response Hsp70-mediated cell protection [45]. Interestingly, NO associated with Hsp70 interaction may modulate WT1 mRNA expression, preventing obstruction-induced cell death during neonatal UUO [10] (Table 1; Fig. 1). This is particularly significant since it is known that Hsp70 is an important cofactor for the function of WT1. Colocalization of WT1 and Hsp70 was evident within podocytes of the developing kidney, and Hsp70 is recruited to the characteristic subnuclear clusters that contain WT1. The amino-terminal transactivation domain of WT1 is required for binding to Hsp70, and the expression of that domain itself is sufficient to induce the expression of Hsp70 through the heat shock element (HSE), suggesting a potential role for this chaperone during kidney development [6] (Fig. 1). The potent induction of Hsp70 by WT1 and the physical association between these two proteins suggests a role for Hsp70 in a cellular differentiation pathway (Fig. 1). Additionally, Zhou et al. investigated whether Hsp72 inhibits TGF-beta-induced epithelial-to-mesenchymal transition (EMT) by modulating Smad expression, activation, and nuclear translocation, and concluded that Hsp72 inhibits EMT in renal epithelial cells primarily by exerting domain-specific effects on Smad3 activation and nuclear translocation [46].

Recently, a strong clinical interest has been generated about the possibility of applying pharmacological agents to induce Hsp70 and prevent renal damage; however, an increased mechanistic understanding of the protective nature of Hsp70 is needed [47]. In particular, further investigation of HSP expression on inflammatory behaviour is required as this could lead to the development of new therapeutic strategies. In this context, we have recently discussed the Hsp70 implications in the renal inflammatory response [48]. To highlight, data provide evidence that the Hsp70 family affords protection via modulation of the inflammatory pathway in the inflammatory response. Recent studies point to a combination of effects including inhibition of apoptosis and inflammation, as primary effectors of Hsp70 action. Additionally Hsp70 via its links with WT1 stabilizes B cell lymphoma 2 (Bcl-2) limiting the potential for cytochrome C release from the mitochondrion and the activation of the intrinsic apoptotic pathway [49]. This is particularly interesting since it is known that nephrogenesis is a highly energy-demanding biological process, with the energy being utilized for renal growth and transport activities. Even more, the loss of mitochondrial Hsp70, an element vital to the protein import process, has been linked to inflammatory alterations [50].

NO/Hsp70 interaction and kidney development

NO, a simple molecule synthesized from L-arginine by NO synthases (NOS), has been identified as playing an important role in regulating adult renal function, and recent studies from our group suggest an even more critical role for NO in the developing kidney. Specifically, it has numerous physiological roles, including the regulation of renal and glomerular hemodynamics, mediation of pressure natriuresis, maintenance of medullary perfusion, blunting of tubuloglomerular feedback (TGF), inhibition of tubular sodium reabsorption, and modulation of renal sympathetic nerve activity (for a review see Vallés and Manucha [51]). Because of its high diffusibility, NO produced in one nephron segment or the renal vessels could affect the function of surrounding structures. Therefore, NO does not need to be produced in a nephron segment to have an effect. The localization of the different sources of NO in the kidney provides a clue as regards how they may act in controlling the renal function.

As mentioned before, the functional integrity of the kidney depends on normal development as well as on physiological cell turnover. And apoptosis induction is essential for these mechanisms (Fig. 1). Apoptosis represents an efficient cellular suicide pathway with characteristics of death in individual cells, induced by physiological and also pathological stimuli with phagocytosis of adjacent cells and without inflammatory response [52]. Nevertheless, based on multiple studies, this concept should be reviewed and possibly expanded.

The ubiquitous distribution of the NO synthases and the remarkable diffusibility and diverse chemical reactivity of NO in biological systems make this molecule unique among the regulators of apoptosis. Moreover, NO could be considered as a bifunctional regulator of apoptosis [53], and NO cytotoxicity has been the topic of intense study; however, a potent antiapoptotic activity of NO has also been proposed [51].

An original antiapoptotic mechanism was proposed for NO inducing Hsp70 (Fig. 1), by means of NO mediated modification in intracellular antioxidants levels [54]. Following this proposal, in UUO rats treated with Losartan, an angiotensin II type 1 receptor (AT_1) antagonist, we have shown decreased tubulointerstitial fibrosis as well as

oxidative stress, both linked to the upregulation of Hsp70 expression [42] (Table 1). Also, using NO donors, we showed enhanced Hsp70 expression and decreased caspase 3 activity in neonatal UUO, while caspase 3 activity was increased in the presence of the specific Hsp70 antibody. In addition, interaction between NOS and Hsp70 was determined by coimmunoprecipitation in cortex membrane fractions, showing an increased ratio of both proteins after rosuvastatin treatment in obstructed kidney; this treatment results in the capacity to prevent both mitochondrial apoptotic pathway and oxidative stress in neonatal early kidney obstruction [55] (Table 1; Fig. 1).

In the neonatal period, a time associated with a hyperactivation of vasoactive systems, NO [9, 56] and Hsp70 [57] seem to play a greater role than in the adult. It could also be implicated in the response to vasoconstrictive stresses, such as perinatal hypoxia, frequently encountered during this period. These findings suggest that the developmental expression of HSP and NO may play a critical and fundamental role in the well-observed tolerance of immature tubules to ischemic or anoxic injury. A previous work has shown a functional WT1/iNOS promoter interaction [8] (Fig. 1). Following this idea of interaction, Hsp70 was proposed as an important cofactor for the function of WT1, suggesting a potential role for this chaperone during kidney differentiation [6]. Moreover, we demonstrated that WT1 mRNA expression is modulated by NO availability and Hsp70 interaction (Table 1; Fig. 1) after neonatal UUO [7]. The WT1 gene acts as a transcriptional activator or repressor depending on the cellular or chromosomal context. Thereby, congenital obstructive nephropathy disrupts normal renal development and causes chronic progressive interstitial fibrosis, which contributes to renal growth arrest, ultimately leading to chronic renal failure. WT1 is downregulated during congenital obstructive nephropathy, leading to apoptosis. Interestingly, NO bioavailability associated with Hsp70 interaction may modulate WT1 mRNA expression (Fig. 1), preventing obstruction-induced cell death during neonatal UUO [10] (Table 1). The latest genetic research has made it possible to characterize many of the complex interactions among the individual components cited, but carrying out new biochemical, molecular, and functional experiments, as proposed by our and other research labs, would allow us to establish a deeper level of commitment among the proteins involved and the potential pathogenic consequences of their imbalance. Specifically, reduced NO release induces Hsp70 expression, mediating beneficial effects against oxidative stress injury, inflammation and apoptosis. Hsp70 may be used as a biomarker linked to inflammation signaling. Elucidating the signaling pathways and the roles of NO and HSPs is relevant to the application of new treatments, such as heat shock and thermal therapy, nitrosylated drugs, chemical chaperones or exercise training [58].

Finally, the functional integrity of the kidney depends on normal development as well as on physiological cell turnover. And apoptosis modulation is essential for these mechanisms. Cumulative evidence suggests that Hsp70 and NO may play a critical and fundamental role in the capacity to modulate both apoptotic pathway and oxidative stress during kidney development. However, the design of experimental protocols to assess renal epithelial functionality in this context could contribute more to the understanding of renal development and alterations.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflicts of interest, financial or otherwise, related to the content of this article.

Ethical approval This article does not contain any studies with human participants performed by any of the authors.

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