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Turmeric-based diet can delay apoptosis without modulating NF-κB in unilateral ureteral obstruction in rats

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Abstract

The unilateral ureteral obstruction (UUO) model of renal injury in rat is characterized by nuclear factor κB (NF-κB) activation and tumour necrosis factor α (TNF-α) production, which induces apoptosis via activation of caspase 8 resulting in cell death. Curcumin, the major component found in turmeric spice, has been reported to provide protection against fibrosis and apoptosis elicited by UUO. This study examined the effects of a turmeric-based diet (5% w/w) on the apoptotic pathway induced by UUO in rats after 30 days of ligation. Administration of a turmeric-based diet demonstrated a significant decrease ($P < 0.05$) in mRNA expression of TNF-α and caspase 8, but not NF-κB, expression, which may contribute to the protective role of the turmeric-based diet. We conclude that a turmeric-based diet can delay apoptosis without modulating NF-κB, so as not to sensitize the mesangial cells to the apoptotic stimuli.

Introduction

Congenital obstructive nephropathy is the primary cause for end-stage renal disease in children. An increasingly used animal model of obstructive nephropathy is unilateral ureteral obstruction (UUO). This model mimics, in an accelerated manner, the different stages of obstructive nephropathy leading to tubulointerstitial fibrosis: cellular infiltration, tubular proliferation and apoptosis (Roth et al 2002).

UUO is characterized by nuclear factor κB (NF-κB) activation, fibrosis and apoptosis (Morrissey & Klahr 1997; Klahr & Morrissey 2000). The activation of NF-κB is a double-edged sword. While needed for proper immune system function, inappropriate NF-κB activation can mediate apoptosis, cell proliferation, differentiation and angiogenesis (Luo et al 2005). NF-κB has both pro- and anti-apoptotic properties and can up-regulate the expression of either cell survival molecules or pro-apoptotic factors, such as tumour necrosis factor α (TNF-α), depending on the surrounding cellular conditions (Karin et al 2002; Misseri et al 2004a). TNF-α is a cytotoxic cytokine that exerts a broad spectrum of responses by binding to two cell-surface receptors, TNFR1 and TNFR2 (Vandenabeele et al 1995), to induce apoptosis through interactions with TNFR1 (Meldrum et al 2003), leading to activation of caspase 8, which in turn triggers caspase 3 activation, resulting in cell death. An increase in NF-κB activation has been observed during renal obstruction (Morrissey & Klahr 1998; Miyajima et al 2003). The direct role of NF-κB in obstruction-induced renal tubular cell apoptosis remains unknown, since NF-κB inhibition has been shown to reduce the expression of the pro-apoptotic TNF-α (Meldrum et al 2003). Meanwhile, other studies reported that NF-κB inhibition can sensitize renal cells to TNF-α mediated apoptosis (Sugiyama et al 1999).

Curcumin, a yellow curry spice derived from turmeric, is widely used to treat various disorders and pathologies (Aggarwal et al 2003). The organic extract of turmeric, a ground powder from the root of the *Curcuma* plant, contains 79–85% curcumin as the major component according to the National Cancer Institute and the Food and Drug Administration (National Toxicology Program 1993). Recent evidence that curcumin exhibits strong anti-inflammatory and antioxidant activities and modulates the expression of transcription factors, cell cycle proteins and signal-transducing kinases and immunomodulation has prompted mechanism-based studies on the potential of curcumin to primarily prevent and treat inflammatory

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diseases (reviewed by Gautam et al 2007). Administration of curcumin has been reported to attenuate renal interstitial inflammation and fibrosis elicited by ureteral occlusion (Kuwabara et al 2006) and angiotensin-II-induced tubular cell apoptosis (Bhaskaran et al 2003). However, the effect of curcumin in protection of mesangial tissue against apoptotic caspase 8 has not been studied in UUO models. Therefore, this study was undertaken to evaluate the protective effect of curcumin against UUO-induced apoptosis.

Materials and Methods

Experimental animals

Thirty male Wistar rats, 250 ± 40 g, were supplied by the Egyptian Organization for Biological Products and Vaccines. Rats were subjected to controlled conditions of temperature (25 ± 2°C) and illumination (12-h light–dark cycle) and allowed free access to normal rat chow diet and water. This protocol was approved by the Animal Care and Use committee of the Biochemistry Department, Faculty of Pharmacy, Beni-Sueif University.

Experimental design

One week after acclimatization, UUO was performed as described previously (Iwano et al 2002). Briefly, after induction of general anaesthesia by intraperitoneal injection of thiopental (100 mg kg^{-1}), the abdominal cavity was exposed via midline incision and the left ureter was ligated at two points with 3-0 silk. The diet was supplemented with turmeric powder (Sekem Co., Cairo, Egypt) in a dose of 5% w/w (n = 10), while the remaining UUO rats were kept on the standard chow diet (n = 10). Sham-operated rats without ureteral ligation (n = 10) were used as a control. After 30 days of the study, blood samples were collected via retro-orbital bleeding for serum separation and stored at −20°C for further analysis. Rats were then killed; the obstructed kidney (OK) and the contralateral unobstructed kidney (CUK) were harvested for mRNA extraction. Histological examination was done for OK.

Creatinine and blood urea nitrogen (BUN)

Creatinine and BUN were determined enzymatically using commercially available kits (Spinreact, Gerona, Spain) and were expressed as mg dL^{-1}.

mRNA determination

About 20 mg of kidney tissue was homogenized, and then centrifuged at 10 000 rev min^{-1}. The supernatant was examined for detection of gene expression. All the data was normalized against β-actin as internal house-keeping gene.

RNA determination

Total RNA was extracted from tissue homogenate by RNA extraction kit supplied by (Promega, Madison, WI) according to manufacturer's instructions and then the extracted RNA was quantitated by spectrophotometer at 260 nm.

Reverse transcription-polymerase chain reaction (RT-PCR) experiments

RT-PCR experiments were carried out on the extracted RNA for detection of the expression of NF-κB, caspase 8 and TNF-α genes.

NF-κB

cDNA was synthesized under the following conditions: 1 μg total RNA was incubated with 5× buffer, 1 mM each dNTP, $MgCl_2$ (5 mM), 50 unit of RNase inhibitor and reverse primer. After denaturation at 65°C for 15 min, 20 U of AMV reverse transcriptase was added, then the reaction tube was placed at 42°C for 1 h, followed by heating to 92°C for 2 min to stop the reaction. Then PCR reaction was performed by adding the PCR mixture to a final volume of 100 μL. The PCR mixture contained 10 mM Tris-HCl pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 10 mM dNTPs mixture, 2.5 U Taq polymerase and 100 μM of each primer. The reaction mixture was then subjected to 40 cycles of 95°C for 1 min, 55°C for 1 min and 72°C for 2 min. After the last cycle, a final extension at 72°C for 10 min was done.

Caspase 8 and TNF-α

Total RNA was reverse transcribed by oligo (dT) 18 primer, reverse transcriptase and RNase inhibitor, then PCR was performed in a total volume of 50 μL. The PCR mixture was formed of PCR buffer, $MgCl_2$, primers, dNTPS and Taq polymerase. The reaction mixture was then subjected to 40 cycles of 95°C for 1 min, 55°C for 1 min and 72°C for 2 min. Primer sequences were as follows. For NF-kB: F: 5′ TAC CAT GCT GTT TTG GTT AC-3′; R: 5′ TCA AGC ACC AAT GAC TTT C-3′. For caspase 8: F: 5′ GTT CAC ACC ATT CTC CTG CC-3′; R: 5′ GAA ACC CCG TCT CTA CTA AA 3. For TNF-α: F: 5′ TCT CAA GCC TCA AGT AAC AAG C-3′; R: 5′ ATG AGG TAA AGC CCG TCA GC-3′.

Agrose gel electrophoresis

All the PCR products of the 3 genes were electrophoresed on 2% agarose gel stained with ethidium bromide and visualized by UV transilluminator.

Gel documentation

The PCR products were semi-quantitated using the gel documentation system (Bio Doc Analyze) supplied by Biometra.

Light microscopic studies

Apoptosis was detected by DNA fragmentation factor-45 (DFF-45) (Widlak 2000). Sections were immuno-stained with anti-rabbit DFF-45 monocolonal antibody using DAKO Lab vision kit. Paraffin sections, 3-μm thick, were mounted on positive slides. They were de-paraffinized, dehydrated, rinsed in tap water, embedded in 3% H_2O_2 in ethanol and rinsed in phosphate buffer. Primary antibody was applied to each slide, 50 μL of antiserum was diluted with 50 μL of distilled water then

diluted antiserum was added to an equal amount of phosphate buffer solution. Secondary antibody was applied. Strept–avidin–biotin and 2 drops of DAB were added. Mayer's hematoxylin was used as a counter stain. Other paraffin sections (3-μm thick) were stained with hematoxylin and eosin (H&E) (Drury & Wallington 1980) as well as Mallory's trichrome (MTC) (Shaffer et al 1987; Satoh et al 2001) and examined under ×400 magnification.

Statistical analysis

All the data are expressed as mean ± s.d. Statistical analyses were performed using SPSS software (SPSS Science, Chicago, IL). Analysis of variance with post-hoc LSD test was performed for comparison between the different groups. $P < 0.05$ was considered statistically significant.

Results

We used the unilateral ureteral obstruction (UUO) model of renal injury in rat, characterized by interstitial inflammatory cell infiltration, activation of NF-κB, as well as caspase 8 in response to the apoptotic stimuli TNF-α (Kaufmann & Hengartner 2001), in addition to fibrosis and apoptosis (Morrissey & Klahr 1997; Klahr & Morrissey 2000).

Kidney function

UUO resulted in significant changes in the routine biochemical function of kidney compared with the sham control group as revealed by a significant increase in both serum creatinine (1.34 ± 0.20 vs 0.90 ± 0.13, $P < 0.05$) and BUN (11.8 ± 1.74 vs 5.6 ± 1.29, $P < 0.05$), while significant amelioration was induced by turmeric-based diet as compared with UUO rats (serum creatinine: 1.1 ± 0.18 vs 1.34 ± 0.20, $P < 0.05$; BUN: 8.2 ± 1.46 vs 11.8 ± 1.74, $P < 0.05$) as illustrated in Table 1.

Detection of TNF-α mRNA

There was a significant increase in TNF-α mRNA expression in the OK up to 212% as compared with the sham group at $P < 0.05$ (Figure 1, 2A), while turmeric-based diet supplementation significantly decreased TNF-α mRNA expression in the obstructed kidney treated with turmeric-based diet (OK-C) by 24% as compared with OK at $P < 0.05$. No significant change was recorded in either CUK or contralateral obstructed kidney treated with turmeric-based diet (CUK-C) in comparison with the sham group.

Table 1 Serum creatinine and BUN in the sham, UUO and UUO-curcumin-treated rats at 30 days of UUO

	Sham	UUO	UUO-curcumin
Creatinine (mg dL^{-1})	0.9 ± 0.13	1.34 ± 0.2$^{\#}$	1.1 ± 0.18*
BUN (mg dL^{-1})	5.6 ± 1.29	11.8 ± 1.74$^{\#}$	8.2 ± 1.46*

All values are mean ± s.d. $^{\#}P < 0.05$ vs sham group; $^{*}P < 0.05$ vs UUO group.

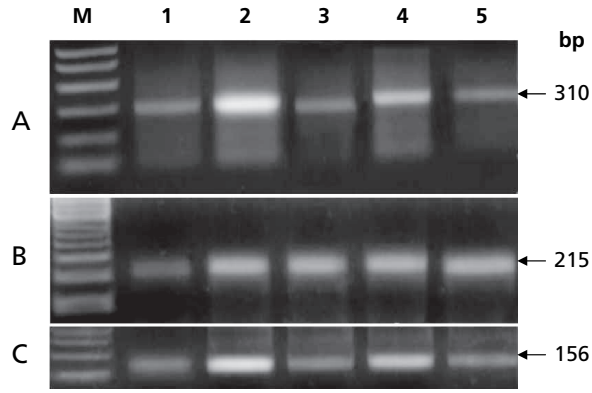

Figure 1 Agarose gel electrophoresis showing gene expression by RT-PCR. Lane M, PCR marker with 100 bp; Lane 1, sham; Lane 2, OK; Lane 3, CUK; Lane 4, OK-C; Lane 5, CUK-C. A, TNF-α, at 310 bp; B, NF-κB at 215 bp; C, caspase 8 at 156 bp.

Detection of NF-κB mRNA

Figures 1 and 2B illustrate a significant increase in NF-κB mRNA level in OK and CUK groups in comparison with the sham group ($P < 0.05$). Meanwhile, turmeric-based diet produced no significant change among OK-C and CUK-C as compared with OK and CUK, respectively, at $P < 0.05$.

Detection of caspase 8 mRNA

There was a significant increase in gene expression of caspase 8 in the OK, up to 179%, in comparison with the sham control group ($P < 0.05$), but supplementation with turmeric-based diet reduced this increment to 69% ($P < 0.05$). Neither CUK nor CUK-C showed significant changes in caspase 8 mRNA levels compared with sham control rats (Figure 1 2C).

Detection of apoptosis

Cleavage of chromosomal DNA into oligonucleosomal-size fragments is a biochemical hallmark of apoptosis (Wyllie 1980). DFF-45 is capable of causing chromosomal DNA fragmentation and is clearly important for DNA fragmentation (Zhang & Xu 2000). Figure 3B, C depicts strong to moderate positive immunoreactivity to DFF-45 in OK and OK-C, respectively, in the glomeruli and dilated tubular cells in comparison with weak positive immunoreactivity in the sham control group (Figure 3A).

Histological examination of renal sections stained with H&E and derived from OK showed intense interstitial cellular infiltration and dilated tubules with flattened epithelium; hyaline cast and cellular debris are seen in some tubules as well as small-sized renal corpuscles as compared with the sham control group (Figure 3D, E). Supplementation of UUO rats with turmeric-based diet resulted in less interstitial cellular infiltration, apparent renal corpuscles and some tubules are relatively dilated and contained epithelial debris (Figure 3F). Staining with MTC showed heavy collagen deposition around the renal tubules and the interstitium in OK group (Figure 3H) when compared with the deposited thin layer of collagen around the

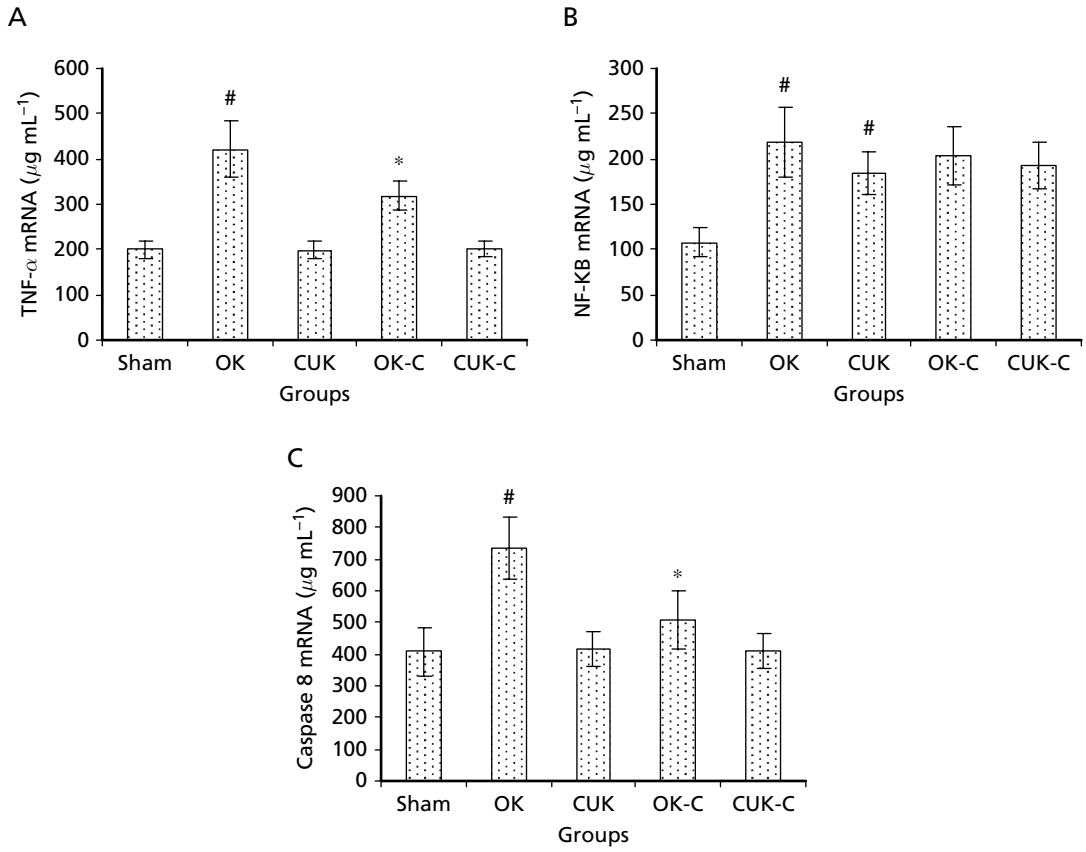

Figure 2 Concentration of PCR products of gene expression (TNF-α mRNA (A), NF-kB mRNA (B), caspase 8 mRNA (C)) in the contralateral and obstructed kidneys of rats with or without turmeric-based diet treatment at 30 days of UUO. Values are means ± s.d., n = 10. $^{\#}P < 0.05$ vs sham; $^{*}P < 0.05$ vs OK.

renal corpuscles and convoluted tubules in the sham group (Figure 3G). Moderate collagen fibres were detected after turmeric-based diet administration (Figure 3I).

Discussion

Upper urinary tract obstruction is a complex injury that results in progressive tubulointerstitial fibrosis and a reduction in renal mass via apoptotic cell death. A number of different inflammatory mediators and growth factors contribute to obstructive renal injury (Misseri et al 2004b). NF-κB activation (Morrissey & Klahr 1997), in addition to an increase in the mRNA levels of TNF- α, TNFR1, caspase 8, and caspase 3, has been documented in rat models of renal obstruction (Kaneto et al 1996; Choi et al 2000; Truong et al 2001). TNF-α has recently emerged as an important mediator of both obstruction-induced renal fibrosis and cellular apoptosis (Guo et al 2001; Misseri et al 2005). TNF-α is a pro-inflammatory, cytotoxic cytokine that is produced from native renal tubular cells in response to injury (Meldrum et al 2001; Misseri et al 2004a), even after 3 days of unilateral ureteral obstruction (Meldrum et al 2006). This completely coincides with our results where TNF-α mRNA expression was only detected in OK in high level in comparison with the sham group. Moreover, the important mediator of obstructive renal injury NF-κB was also expressed (Morrissey & Klahr 1997; Tashiro et al 2003). TNF-α is a cytotoxic cytokine that induces apoptosis in many cells, including renal tubular cells, through interactions with its membrane-bound receptor, TNFR1 (Chinnaiyan et al 1995; Meldrum et al 2001). Maeda et al (2003) demonstrated that systemic release of TNF, which induces hepatocyte apoptosis and liver failure, requires both TNFR1 (mainly) and TNFR2 for maximal apoptosis. Furthermore TNFR1-deficient mice were found to be resistant to this apoptotic effect (Maeda et al 2003).

TNF-α induced effects are mediated by TNFR1. The intracellular domain of the latter is characterized by the presence of an 80-amino-acid-long death domain (DD), which is responsible for the generation of the cytotoxic death signals, as well as for the activation of the transcription factor NF-κB. A similar DD is found in several cytoplasmic signalling proteins, including TNFR-associated DD protein (TRADD) and Fas-associated protein with a death domain (FADD). Triggering of TNFR1 by TNF-α leads to clustering of pre-assembled TNF-R complexes (Chan et al 2000) and recruitment of TRADD to the TNF-R via a homotypic DD–DD interaction. TNF-induced signalling pathways leading to the activation of NF-κB and apoptosis bifurcate at the level of TRADD (Hsu et al 1995). Another protein that interacts with the DD of

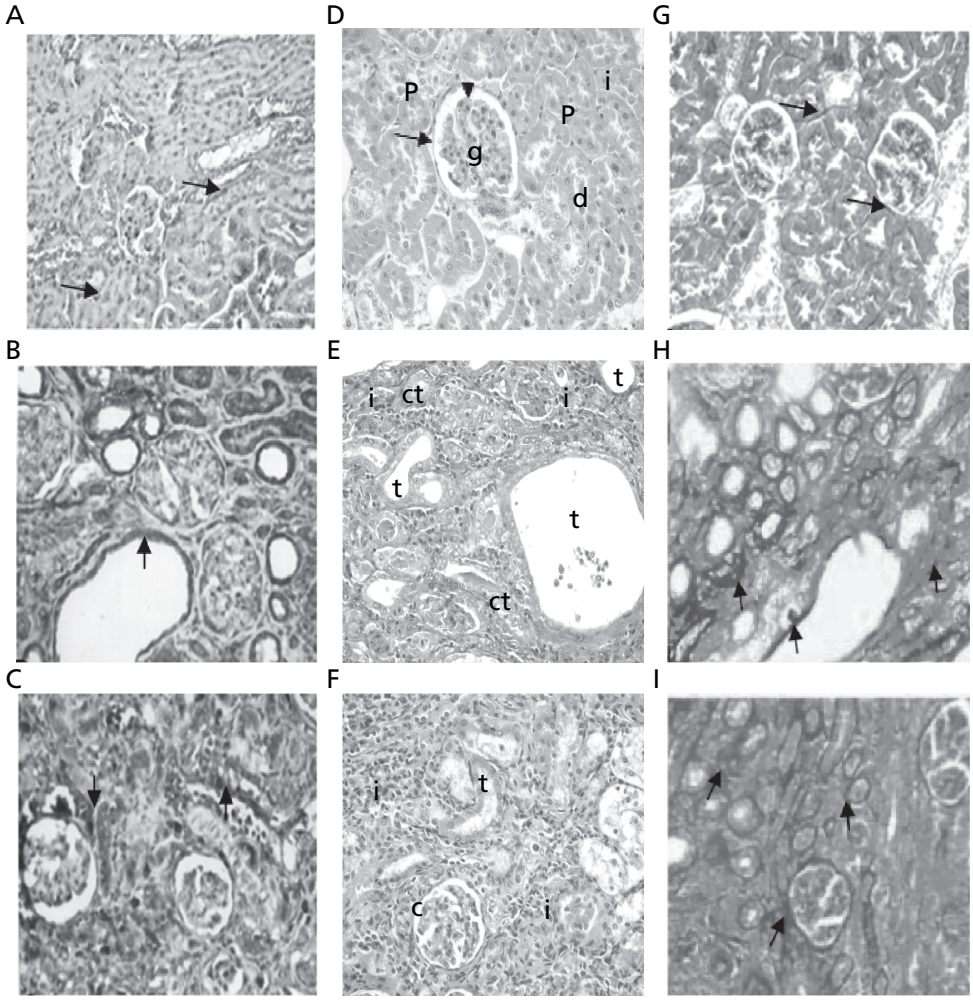

Figure 3 A photomicrograph of renal cortex sections showing: minute positive immunoreactivity to DFF-45 (⟶) in sham group (A); intense positive immunoreactivity in the dilated tubular cells (→) in OK group (B); mild positive immunoreactivity in the renal tubular cells (⟶) in the OK-C group (C); a renal corpuscle consisting of a glomerulus (g) and Bowman's capsule with its visceral (▶) and parietal layers(⟶), proximal convoluted tubules (p) and distal convoluted tubules (d) in a sham group (D); intense interstitial mononuclear cellular infiltration (i), dilated tubules with flattened epithelium (t) and collapse of some tubules (ct) in OK group (E); renal corpuscle (c) more or less similar to group A, mononuclear cellular infiltration (i), some tubules relatively dilated and containing debris (t) in OK-C group (F); thin layer of collagen fibres (⟶) around renal corpuscles and the convoluted tubules in sham group (G); heavy collagen deposition around the renal tubules and in the interstitium (⟶) in OK group (H); moderate collagen fibres around the renal corpuscle and renal tubules (⟶) in OK-C group (I). ×400 magnification.

TRADD is FADD (Hsu et al 1995). This protein also functions as an adapter protein, and contains two functionally and structurally distinct domains: a C-terminal DD that is necessary for its recruitment to the TNFR1 complex and an N-terminal death effector domain (DED) that promotes activation of a downstream proteolytic cascade through binding of the homologous DEDs of procaspase 8. Caspases are a family of aspartate-directed cysteine proteases that are essential effector molecules in pro-apoptotic pathways, which are initiated in response to several death stimuli such as TNF-α (Budihardjo et al 1999). Indeed, TNF-α induces apoptotic renal tubular cell death via death receptor signalling and caspase 8/3 activation (Misseri et al 2005). Synchronization of TNF-α and caspase 8 mRNA expression was only recorded in the injured kidney of the UUO animal model (Chinnaiyan et al 1995; Hsu et al 1995; Meldrum 1998). Caspase 8 activation, in turn, triggers caspase 3 activation, resulting in cell death (Chinnaiyan et al 1995; Hsu et al 1995). It is noteworthy that previous studies using mouse embryofibroblasts (MEFs) deficient in caspase 8 showed resistance to TNF-α-induced apoptosis (Varfolomeev & Ashkenazi 2004). Interestingly, it has been reported that kidneys obstructed in the presence of TNF-α neutralization had a marked reduction in NF-κB activity, approaching sham treatment levels. This finding identifies TNF-α as an important mediator of NF-κB activation during renal obstruction (Meldrum et al 2006).

We found that NF-κB is activated in both kidneys, the OK and CUK, while caspase 8 is increased in the OK only leading to its programmed cell death. Curcumin administration stopped the signalling death molecule caspase 8 revealing an

anti-apoptotic role of curcumin. This has been achieved without modulating the NF-κB activity either in OK or CUK. Moreover, histopathological examination showed apparent renal corpuscles in the OK cortex. This finding may be explained by the selective inhibition of TNF-α (Giri et al 2004), the pro-apoptotic stimuli that triggers the caspase cascade. It seems that the deletion of caspase 8 and stopping the cell death program is very beneficial where this can give the chance for NF-κB to act as an anti-apoptotic molecule to reconstruct the kidney tissues as seen in the hisopathological estimation. This finding coincides with the concept of Sugiyama et al (1999) who stated that the blockade of NF-κB selectively sensitized mesangial cells to TNF-α-induced apoptosis and that sensitization was still demonstrable in the presence of survival factors in serum, which are known to protect mesangial cells against many pro-apoptotic stimuli. Although highly speculative, the possibility is raised that selective sensitization to TNF-α might enable the combination of this cytokine and the NF-κB blockade to be used as a molecular knife to pare away excess mesangial tissue cells where these threaten accumulation of abnormal extracellular matrix and progression of glomerular inflammation to a functionless scar (Sugiyama et al 1999). Obviously this may explain how curcumin can be very effective to stop caspase-8-mediated apoptosis without affecting NF-κB; the data also emphasize that attempts to ameliorate inflammation by inhibition of NF-κB could have undesirable consequences where NF-κB activation serves as a primary mechanism to protect cells against an apoptotic stimulius (Wang et al 1998). One possible explanation relating to the NF-κB activation is TRADD interaction with TNF-receptor-associated factor 2 (TRAF2), which is required for an NF-κB-independent signal that protects against TNF-α induced apoptosis (Yeh et al 1997). Activation of NF-κB induces the expression of several proteins, including anti-apoptotic proteins, that might interfere with the pro-apoptotic pathway at several steps (Heyninck & Beyaert 2001). This may transfer us to another area that may explain why NF-κB is activated in the CUK to a level approaching the OK although there is no renal injury and, in consequence, apoptotic stimuli in this mesangial cell. We think that the meaningful activation of NF-κB is linked to the compensatory overload, which has been performed by the CUK to face the large demands required by this intact kidney. Further investigations are needed to answer many questions concerning the NF-κB activation and its role in OK protection, where it could be involved in triggering certain anti-apoptotic molecules in OK or angiogenic factors in the CUK to perform its extra overload during UUO.

Conclusion

We conclude that UUO can lead to activation of NF-κB in both OK and CUK. The OK is considered as an inflammatory medium that triggers apoptosis, and the beneficial effect of curcumin administration is represented by its ability to delay apoptosis without modulation of NF-κB to not sensitize the mesangial cells to apoptotic stimuli in the absence of NF-κB. Moreover, NF-κB has been demonstrated to be correlated with improvement of the OK architecture. Also we think that the CUK can perform the extra load by the compensatory increase in NF-κB despite the absence of injury and hence to switch on a series of reactions that may be considered protective.

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