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The Role of Tumor Necrosis Factor Alpha in Regulating the Expression of Tamm-Horsfall Protein (Uromodulin) in Thick Ascending Limbs during Kidney Injury

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Abstract

Background—Tamm-Horsfall Protein (THP) is a glycoprotein expressed exclusively by cells of the thick ascending loop (TAL) of Henle. THP has a protective role in acute kidney injury (AKI), and its expression is downregulated in the early stages of injury. Tumor necrosis factor alpha (TNF α) is a cytokine endogenously expressed by the TAL and is also induced by AKI. Therefore, we hypothesized that TNF α is a key regulator of THP expression.

Methods—We used a mouse model of AKI (ischemia-reperfusion injury, IRI) and a cell culture system of a TAL cell line (MKTAL).

Results—We show that TNF α is upregulated by TAL cells early after AKI in vivo. The expression of THP and its transcription factor Hepatocyte nuclear factor 1 β (HNF1 β) were concomitantly decreased at the peak of injury. Furthermore, recombinant TNF α inhibits significantly, and in a dose-dependent manner, the expression of THP, but not HNF1 β in MKTAL cells. Interestingly, neither TNF α neutralization nor genetic deletion of TNF α increased THP or HNF levels after injury in vivo.

Conclusion—Our data suggest that TNF α can inhibit the expression of THP in TAL cells via an HNF1 β -independent mechanism, but the down-regulation of THP expression in the early AKI does not depend on TNF α . We propose that TNF α regulates THP expression in a homeostatic setting, but the impact of TNF α on THP during kidney injury is superseded by other factors that could inhibit HNF1 β -mediated expression of THP.

Keywords

Uromodulin; Ischemia-reperfusion; Cytokine

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Disclosure Statement

The authors declare no competing interests.

Introduction

Tamm-Horsfall Protein (THP) is one of the most abundant urinary proteins, expressed and produced exclusively by cells of the thick ascending limbs (TAL) of Henle [1, 2]. The last decade has experienced a surge of knowledge linking THP or mutations in THP to many physiological or pathogenic processes in the kidney [1, 2]. We recently showed that THP is protective against acute kidney injury (AKI) using a mouse model of renal ischemia reperfusion injury (IRI) [3–5]. The presence of THP in vivo was essential to suppress the ensuing inflammatory response after IRI. Interestingly, we and others showed that THP expression is inhibited at the peak of injury [3, 6, 7], suggesting that early AKI is a state of relative THP deficiency. During recovery, THP expression is significantly increased and this suggests that this glycoprotein is essential to mitigate the propagation of inflammation and restore homeostasis in the kidney after injury [3]. It is important to note that limited data is available on what factors regulate THP expression [1]. In fact, solid data exists only on Hepatocyte nuclear factor 1 beta (HNF1 β), which is a major transcription factor that binds upstream DNA elements of the THP gene, and enhances positively its expression [8].

Tumor Necrosis Factor alpha (TNF α) is an inflammatory cytokine that is induced in AKI and is implicated in the pathogenesis of kidney injury [9–12]. TNF α itself can lead to cytotoxic effects and to the activation of a pro-inflammatory cascade [9, 11]. However, TNF α is also endogenously expressed in TAL [13], and is thought to affect the transport properties of this tubular segment [14]. Since THP may also have a regulatory effect on the function of TAL, it is possible that TNF α could regulate THP expression, especially during kidney injury. Therefore, we hypothesized that TNF α inhibits the expression on THP during the early phase of AKI, thereby contributing to the pathogenesis of this disease. This study was undertaken to test this hypothesis, in the context of understanding the regulation of THP expression in AKI, using an ischemia-reperfusion model of AKI in mice in conjunction with in vitro experiments.

Methods

Animal Surgery

Animal experiments and protocols were approved by the Indiana University Animal Care and Use Committee. 8–10 weeks old mice 129/SvEv (Taconic) were used as previously described [5, 15, 16]. TNF α knockout animals and their congenic C5BL6 strain were from Jackson labs. Ischemia reperfusion surgery using bilateral renal pedicle clamping was as previously described [5, 17]. We used a 22, 30, or 35 min ischemia times to induce varying severity of kidney injury, followed by 6, 24, or 48 h reperfusion based on previous studies [5]. In all TNF α neutralization experiments, we used a 30-minutes clamp time [5]. Because TNF $\alpha^{-/-}$ mice were on C5BL6 background, we used a 35 min clamp time to induce significant injury based on previous studies [17]. Sham surgery consisted of an identical procedure without application of the micro aneurysm clamps. All animals received subcutaneous analgesic (buprenorphine 0.1 mg/kg) at the end of the surgery and every 12 h for 48 h or until euthanasia. Serum creatinine was measured using capillary electrophoresis [18], performed at the UT Southwestern O'Brien Center of Excellence.

TNF α neutralization was done using 200 μ g of intra-peritoneal injection of TNF α neutralizing antibody (R&D) dissolved in 1 cc PBS compared to IgG or PBS using a previously described method [4]. Two protocols were used where TNF α Ab was given 4 h before or at the time of surgery, yielding similar results (data not shown).

Immunofluorescence Confocal Microscopy

Protocols for immunostaining vibratome sections were previously reported [5, 19]. Confocal microscopy was done using an Olympus Fluoview laser scanning confocal microscope as previously described [4, 20]. We used primary antibodies against HNF1 β (Sc-22840, H-85, Santa Cruz), THP (AbD Serotec cat#8595–0054), and TNF α (Novus, NBPI-19532).

Western Blot for THP

Western blot was performed as was described previously [3, 19]. Blocking was done using 5% nonfat dry milk. THP was detected using a sheep anti-human THP (1: 500; AbD Serotec cat#8595–0054) primary, and a horseradish peroxidase-labeled rabbit anti-sheep (1:2,000; Upstate) secondary antibody. Blots were stripped and re-probed for actin using mouse anti-actin (1:1,000; Millipore (cat#MAB1501) and a goat anti-mouse secondary antibody (1:50,000). Bands were detected by enhanced chemiluminescence (ECL, Amersham Corp.). Protein extract from THP^{-/-} kidneys [3] were used as negative controls. Band densitometry was performed using ImageJ (NIH) and measurements are reported after normalization to actin.

MKTAL Cell Cultures and TNF α Dose Response Experiments

MKTAL is a thick ascending limb cell line that was isolated and characterized by Bourgeois et al. [21]. Cells are cultured in DFS, which consists of 1:1 mixture of DME (Gibco #10567–014) and Ham's F12 (Gibco #31765–035) medium containing 5% FBS (Lonza BioWhittaker #14–502F). Experiments were performed when cells were 70–80% confluent. All experiments were performed in triplicates, and representative experiments are shown for each experimental condition. TNF α (R&D Systems #410-MT-010/CF) was added at 1, 10, and 20 ng/ml as indicated. Control wells without TNF α were used for each time point. After 2, 6, or 24 h of incubation, flasks from each group were aspirated, trypsinized, and harvested as above.

Real-Time PCR

RNA extraction from whole kidneys and from MKTAL cells was done using TriReagent (Ambion, AM9738) according to the manufacturer's protocol. Purified RNA was treated with DNase 1 (AB #AM2222) and subsequently reverse-transcribed using High Capacity cDNA Reverse Transcription Kit (AB #4374966). Real-time PCR was performed on the equivalent of 10 or 20 ng RNA in Applied Biosystems (AB) Step One Plus using TaqMan Gene Expression Assays also from AB (THP: Mm00447649_m1; MCP-1: Mm99999056_m1, TNF α : Mm00443258_m1; HNF1 β : Mm00447459_m1). Cycling parameters were: 50°C for 2 min, then 95°C for 10 min followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. We verified that the levels of endogenous controls (GAPDH) were not affected by changes in experimental conditions (CT changes <0.5). All expression was

normalized to endogenous controls and reported as fold change compared to control using the delta-delta CT method, according to the manufacturer's instructions.

Statistical Analysis

Values of each experimental group are reported as mean $\pm$ standard error. A two tailed t test was used to examine the difference in means for continuous data. Statistical significance was determined at the 0.05 significance level.

Results and Discussion

Changes in THP Expression in the Outer Medulla after Ischemia Reperfusion Injury (IRI)

Previous work from our lab and others showed that THP mRNA is significantly downregulated at the peak of kidney injury in rat and mice models [3, 6, 7]. In the current study, we aimed to confirm these previous reports and investigate in more detail the changes in THP protein after severe IRI (fig. 1). Using immuno-fluorescence confocal microscopy, we showed a significant decrease in THP expression 24 h after IRI in TAL (fig. 1c, d) compared to sham (fig. 1a, b). Furthermore, using western blot on total kidney lysates, we confirmed the decrease in THP protein after injury (fig. 1e). We also performed real-time PCR on total kidney RNAs to measure changes in THP mRNA expression in mice 6 and 24 h after IRI surgery as compared to sham mice. As shown in figure 1f, THP expression was significantly decreased 24 h after IRI, which correlates with the protein findings in figure 1d, e. Therefore, both THP message and protein are significantly inhibited 24 h after IRI, which coincides with the peak of injury. These findings are consistent with previous reports [3, 6, 7], with the added knowledge that the reduction in THP protein, especially in total kidney lysates, is more evident with severe injury (fig. 1e) compared to less severe injury as we reported previously [3]. In fact, the level of THP in total kidney lysates depends on the rate of THP production by TAL, the half-life of THP (reported to be rather short: about 9 h in rabbit and 16 h in human; data in mouse not available [2]), and the successful excretion of urinary THP out of the kidney by the flow of urine. Therefore, it is expected that the oliguria induced by IRI will retain THP in the tubular lumen or bound to urinary casts and attenuate the decrease in THP protein measurements after IRI. However, our current data suggest that the decrease in THP production by TAL that occurs with severe injury is so profound, that it also reduced THP levels in total kidney lysates. Of note that we previously showed that the THP expression rebounds during recovery (starting at 48 h after IRI) and THP levels are significantly increased during that time point [3].

Hepatocyte Nuclear Factor 1 β and THP Expression in Ischemia Reperfusion Injury

It is known that HNF1 β , a transcription factor, binds DNA element of the UMOD gene and positively regulates its transcription [8]. Therefore, we hypothesized that the level and nuclear translocation of this transcription factor could serve as a useful surrogate for HNF1 β -mediated transactivation of the THP gene. Using confocal immuno-fluorescence microscopy, we found that in sham kidneys, HNF1 β was localized to TAL primarily in the cytoplasm (fig. 2a–d). HNF1 β staining was significantly decreased at 24 h after ischemia. At 48 h, HNF1 β expression was significantly increased and localized to the nuclei of TAL (2I–L) and proximal tubular segments. In figure 2 Panel M, we also measured the changes in

HNF expression in total kidneys extracts after IRI (with increasing severity of injury, 22 and 30 min clamp times) compared to sham. Regardless of injury severity, there was an initial increase in HNF1 β mRNA level at 6 h after injury, followed by a significant decrease at 24 h, which correlated with the protein level in 2 E–H. A recovery at 48 h was observed for HNF1 β mRNA level, which coincided with the increase in protein level at that time point. Although the apparent increase in HNF1 β staining at 48 h appears disproportionately elevated compared to the mRNA, this could be due to the nuclear translocation/concentration of HNF1 β and/or a post-transcriptional effect. A lower magnification (20 $\times$ objective) of the changes of HNF1 β within the outer medulla is also provided in figure 3. Taken together, these data show that the level of HNF1 β is significantly decreased at the peak of injury, which correlates with the decrease in THP expression, and suggests that the inhibition of THP at the peak of injury may be HNF1 β -dependent.

Our study shows that with recovery, HNF1 β translocates to the nuclei of TAL, which could explain partly the recovery of THP described previously [3]. However, an interesting finding is that HNF1 β nuclear translocation appears to be a generalized phenomenon because it also occurs in proximal tubules. This supports previous findings that HNF1 β could be an important factor in the overall recovery of various renal tubular cells from AKI [22, 23].

Upregulation of TNF α Expression, and Localization to TAL Segments in the Outer Medulla after Ischemia Reperfusion Injury

Tumor Necrosis Factor alpha (TNF α) is an inflammatory cytokine that is induced in AKI and is implicated in the pathogenesis of kidney injury [9–12]. TNF α itself can lead to cytotoxic effects and to the activation of a pro-inflammatory cascade [9, 11]. However, TNF α is also endogenously expressed in TAL [13], and is thought to affect the transport properties of this tubular segment [14]. Since THP may also have a regulatory effect on the function of TAL, it is possible that TNF α could inhibit THP expression at baseline and especially at the peak of AKI. Therefore, we studied TNF α expression in kidney sections from mice after IRI or sham surgeries using immuno-fluorescence confocal microscopy. As shown in figure 4, TNF α expression started to increase 6 h after IRI, and localized predominantly to TAL and less significantly to S3 segments in the junction area between outer and inner stripes within the outer medulla (fig. 4b). TNF α expression increased even further at 24 h after IRI (4C). Using real-time PCR, we also measured the changes in TNF α expression in total kidney extracts after IRI compared to sham (fig. 4d). In IRI 22 min, TNF α mRNA began to increase 6 h after moderate injury and peaked 24 h after IRI (5.6 $\pm$ 1.0 fold increase vs. sham, p < 0.05). At 48 h, TNF α expression decreased, consistent with recovery from AKI. In more severe injury (IRI 30 min), TNF α peak was significantly higher and occurred earlier compared to moderate injury. Taken together, this data shows that TNF α is significantly increased in TAL segments at the peak of injury, which coincides with the inhibition of THP expression.

TNF α as an Inhibitor of THP Expression in TAL Cells

To determine whether TNF α itself caused downregulation of THP expression, we incubated various concentrations of TNF α (1, 10, and 20 ng/ml) with MKTAL cells (an established thick ascending limb cell line [21]), and studied THP mRNA expression using real-time

PCR at various time points after incubation (2, 6 and 24 h). To verify the reactivity of cells to TNF α , we concurrently studied the expression of MCP-1 (Monocyte Chemoattractant Protein-1), a macrophage chemo-attractant that can be induced by TNF α [12]. There was no significant change in THP expression after 2 h of incubation with TNF α in all concentrations used (fig. 5a). At 6 h, we detected a significant decrease in THP expression, which was comparable in all concentrations used (0.6–0.65 fold decrease vs. control). At 24 h, we observed recovery of THP expression with the lowest TNF α concentration used (1.04 ± 0.10 fold change vs. control). A partial recovery was seen with the intermediate (e.g., 10 ng/ml) TNF α concentration (0.73 ± 0.02 fold change, $p < 0.05$). In contrast, no recovery but rather a further decrease was seen with the highest TNF α concentration used (0.40 ± 0.04 fold change, $p < 0.05$). Conversely, MCP-1 was induced significantly in all the time points. The increase was proportional to the concentration of TNF α used (fig. 5b).

Figure 5c also shows the level of HNF1 β mRNA in MKTAL cells treated with TNF α . Although HNF1 β levels were slightly increased at 2 h after TNF α treatment, these levels were unaffected by TNF α in all subsequent time points, suggesting that TNF α has no impact of HNF1 β levels in TAL cells.

The identification of TNF α as an inhibitor of THP expression in TAL cells is novel finding of this study. These findings could be relevant to understand regulatory processes within TAL, especially in view of data by Ferreri and colleagues, that TNF α is endogenously expressed by TAL [13, 14]. Recently, Mutig et al. showed that THP could play a facilitative role in the proper functioning of NKCC2 [24]. Therefore, THP inhibition by TNF α could alter the function of NKCC2; this also supports the recent findings by Battula et al. that TNF α is an endogenous inhibitor of NKCC2 [14]. We acknowledge that using a transformed cell line such as MKTAL cells has its limitations, especially pertaining to the applicability of the results to primary cells or to an in vivo setting. Therefore, we also investigated THP expression in kidneys from sham TNF α knockout mice, compared to kidneys from the corresponding congenic background strain. Figure 5d shows a noticeable trend toward increase in THP expression in TNF $\alpha^{-/-}$ compared to TNF $\alpha^{+/+}$ kidneys ($p = 0.08$), suggesting that TNF α may be an endogenous inhibitor of THP expression.

The Effect of TNF α Inhibition or Genetic Deletion on THP and HNF1 β Expression during Kidney Injury in vivo

To determine the importance of TNF α in regulating THP expression after AKI in vivo, we neutralized TNF α in mice subjected to renal IRI using a specific neutralizing antibody as described in methods. Figure 6 shows that TNF α neutralization did not affect kidney injury, as indicated by serum Cr level (6A), nor did it change the level of THP mRNA (6B) and protein (6C). Similarly, HNF1 β expression was not affected by TNF α neutralization (6B). To verify that the lack of an effect was not due to incomplete TNF α neutralization, we studied kidney injury and THP expression in TNF $\alpha^{-/-}$ and TNF $\alpha^{+/+}$ mice (fig. 6d). Surprisingly, TNF $\alpha^{-/-}$ mice sustained more kidney injury than their wild type controls at 24 h post IRI (fig. 6d, left panel). THP expression was also significantly lower in TNF $\alpha^{-/-}$ (fig. 6d, right panel), suggesting that its expression predominantly correlates with the severity of injury. It is important to acknowledge, in interpreting the data, that genetic variability

between C57BL6 and 129/SvEv could be a variable in determining the response to injury and THP expression. However, the fact that both THP inhibition in 129/SvEv or genetic deletion in C57BL6 did not increase THP expression after injury strongly supports the conclusions reached.

Taken together, these studies uncover TNF α as an inhibitor of THP expression in TAL cells via an HNF1 β -independent pathway. However, it appears that TNF α may not be the major regulator of THP expression after IRI, and that TNF α -independent factors supersede TNF α in regulating THP expression in vivo, possibly via an HNF1 β -dependent fashion. Furthermore, our data also highlight the complex role of TNF α in AKI. It appears that the outcome of TNF α inhibition post AKI depends on the type of injury. It is beneficial in septic AKI [9] and in cisplatin nephrotoxicity [12]. However, it is harmful in tunicamycin toxicity [25], and to our knowledge, this is the first study on TNF α inhibition in mouse IRI. Although TNF α inhibition was protective in rat IRI [11, 26], our data suggest that inhibition of TNF α may not be acutely beneficial in mouse renal ischemic injury. Interestingly, similar discrepancies for p53 inhibition were found between the rat and mouse ischemic injury [27], suggesting a difference in the nature of ischemic injury between the two species. The reasons for the variability in the acute actions of TNF α between mouse and rat will need further investigations.

In conclusion, we showed that TNF α can inhibit the expression of THP in TAL via an HNF1 β -independent mechanism, but the downregulation of THP expression in the early phases of AKI does not depend on TNF α . We propose that TNF α inhibits THP expression in homeostatic settings, but the impact of TNF α on THP during kidney injury is superseded by other factors that could inhibit HNF1 β -mediated expression of THP.

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Fig. 1.

Inhibition of THP expression at the peak of kidney injury. Representative confocal immunofluorescence images (20× objective) of outer medulla sections from kidneys after sham (**a, b**) and IRI surgeries (30 min clamp) with 24 h reperfusion (**c, d**) stained for THP (red). Oregon green-phalloidin was used to identify S3 segments. Note the decrease expression of THP after IRI. asterisk in **c** show casts that stain positive for THP. **e** Shows western blot for THP and actin control on total kidney lysates from sham and IRI mice. Relative Protein quantitation with band densitometry normalized to actin shows a significant decrease of THP in IRI kidneys compared to sham ($p < 0.05$). Bar graphs in **f** show changes of THP mRNA after IRI at 6 and 24 h recovery measured as fold increase compared to sham reference using real-time PCR.* denotes statistical significance compared to sham.

Fig. 2.

HNF1 β expression and nuclear translocation after IRI. Representative confocal immunofluorescent images (60 $\times$ objective) of outer medulla sections from kidneys after sham (a–d) and IRI surgeries (22 min clamp) with 24 or 48 h reperfusion (e–h and i–l, respectively) immunostained for HNF1 β expression (red channel). Oregon green-phalloidin was used to identify S3 segments (green channel), and DAPI stain was used for nuclei (blue channel), with merged images shown on the right column. Thick ascending limbs (TAL) were marked by the letter T. In sham, HNF1 β expression was predominantly seen in TAL in

a cytoplasmic pattern (arrows in **a**). 24 h after IRI, HNF1 expression was decreased. However at 48 h after IRI, there was a widespread upregulation of HNF1 β in a nuclear distribution seen not only in TAL, but also in S3 segments. Asterisk in **J** mark injured S3 segments. Bar graphs in **m** show changes of HNF1 β mRNA after IRI with increasing severity (22 and 30 min clamp times) measured as fold increase compared to sham reference using real-time PCR. * denotes statistical significance compared to sham.

Fig. 3. HNF1 β expression viewed under lower magnification. Representative confocal immunofluorescent images (20 $\times$ objective) of outer medulla sections from kidneys after sham (a) and IRI surgeries (22 min clamp) with 24 or 48 h reperfusion (b, c, respectively) immunostained for HNF1 β expression (red channel). Oregon green–phalloidin was used to identify S3 segments (green channel), and DAPI stain was used for nuclei (blue channel). Thick ascending limbs (TAL). S3 (proximal tubular segments).

Fig. 4.

TNFα expression and localization after AKI. **a–c** Representative, confocal immunofluorescence microscopic images (20× objective) from kidney sections of sham control (**a**) or post-IRI (22 min clamp time) at 6 (**b**) and 24 h (**c**). TNFα was stained red and brush border stained green with Oregon phalloidin. While there was minimal TNFα expression in sham, its expression increased 6 h after IRI predominantly in thick ascending limbs (TAL) and less significantly in S3 (**b**). TNFα expression increased even further at 24 h after IRI. Bar graph in **d** showed changes of TNFα mRNA after IRI with increasing severity measured

as fold increase compared to sham reference using real-time PCR. * denotes statistical significance vs. sham, † denotes significance versus IRI 22 min, ‡ denotes significance compared to IRI 6 h ($p < 0.05$).

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Fig. 5.

TNFα as an inhibitor of THP expression in MKTAL cells. **a–c** Changes in THP, MCP-1 and HNF1β mRNA, respectively, after incubation of cultured MKTAL cells with various concentrations of TNFα for 2, 6 and 24 h. There was no change in THP mRNA at 2 h, but a comparable decrease was seen at 6 h in all experimental conditions (**a**). At 24 h, a further decrease in THP mRNA was seen with the highest concentration of TNFα used (20 ng/ml), whereas a graded recovery was seen with the lower TNFα concentrations. Conversely, MCP-1 mRNA was elevated in all time points, peaking at the 6 h, and proportional to the concentrations of TNFα used. There was a slight increase in HNF1β mRNA at 2 h, but HNF1β mRNA remained comparable to controls in subsequent time points. Bar graphs in **d** show baseline changes in THP mRNA expression from sham TNFα^{+/+} and TNFα^{-/-} total kidney RNA extracts (n = 4–5 per group). There was a trend towards higher THP expression in TNFα^{-/-} kidneys.

Fig. 6.

TNFα neutralization or deficiency, kidney injury and THP expression. **a** show changes at 24 h after IRI in serum Creatinine (serum Cr) compared to baseline before surgery, in mice (n = 5, each group) treated with TNFα neutralizing antibody, non-specific IgG or PBS at the time of surgery. **b** shows changes in THP and HNF1β mRNA in these mice at baseline and after IRI. TNFα neutralization had no significant effect on the level of serum Cr, THP mRNA or HNF1β mRNA as compared to IgG or PBS. The level of THP protein shown in **c**, measured by western blot, was also not different 24 h after IRI between IgG or TNFα antibody treated mice. **d** shows changes of serum Cr (left axis) and THP mRNA in TNFα^{-/-} and TNFα^{+/+} mice 24 h after IRI (n = 5 per group). TNFα^{-/-} had more severe injury as denoted by a significantly higher serum Cr. In addition THP mRNA was significantly lower in TNFα^{-/-} compared to TNFα^{+/+}, suggesting that THP expression correlated more with the severity of injury than the status of TNFα.