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Reduction in the anti-adherence activity of Tamm-Horsfall protein with increasing concentration of calcium

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Summary. Tamm-Horsfall glycoprotein (THP), at concentrations occurring in normal urine, was demonstrated to show anti-adherence activity for type-1 fimbriated *Escherichia coli*. Urine also showed anti-adherence activity, and urine from which the THP was precipitated showed a significant drop in activity. The addition of calcium to the incubation medium with THP, at concentrations normally found in the urine, had no effect on the anti-adherence activity of THP. However, concentrations of calcium higher than those normally occurring in the urine significantly decreased the anti-adherence activity of THP. It is suggested that individuals with above normal concentrations of calcium in the urine may be at increased risk for urinary tract infections since the protective effect of THP is compromised.

Key words: Urinary tract infection – Tamm-Horsfall protein – Calcium-Adherence

Tamm-Horsfall protein (THP), a renal glycoprotein, is the most abundant protein in normal urine. Orskov et al. [9] demonstrated that type-1 fimbriated *Escherichia coli* are trapped by THP and suggested this prevents attachment of the bacteria to uroepithelial cells, thus preventing infection. Kuriyama and Silverblatt [7] demonstrated, via electron microscopy, that THP forms a pseudocapsule around bacteria with type-1 fimbriae, and in the absence of the fimbriae no capsule was formed. The binding of THP to the fimbriae is presumably due to mannose contained in the THP and is supported by their observation that methyl mannoside reduced the affinity of purified fimbriae for THP on an affinity column. Dulawa et al. [30] demonstrated that bacterial adherence, mediated by mannose-sensitive type 1 fimbriae, can be inhibited by THP. The adherence was specific and dose dependent, with high concentrations inhibiting adherence while low concentrations showed only a marginal inhibition and in some preparations even enhanced adherence. Duncan [4] also observed that at concentrations greater than 30 μg/ml THP inhibited bacterial adherence. However, at lower concentrations THP stimulated bacterial adherence. Since type-1 fimbriated *E. coli* have been isolated from patients with lower urinary tract infections [6], the binding of THP of these bacteria may represent an important host-defense mechanism in bacterial clearance of the urinary tract. In a previous publication we demonstrated that if the diet is supplemented with calcium, there is an increase in the concentration of calcium in the urine, and when bacteria and uroepithelial cells are incubated in this urine there is a corresponding increase in bacterial adherence [1]. In this study we demonstrate that calcium ions, at concentrations higher than normally occurring in the urine, strongly inhibit the anti-adherence effect of THP. This observation has implications for individuals on calcium supplements, suggesting a decrease in the normal anti-adherence effect of THP in the urine and implies an increased risk for urinary tract infections.

Materials and methods

E. coli strain 1401 was used in this study. The organism was obtained from the microbiology laboratory of Alliance City Hospital and was isolated from a patient with diagnosed urinary tract infection. API 20E strips (Fisher Scientific, Pittsburgh, Pa./USA) were used to confirm the identity of the isolate. The organism was shown to express type-1 fimbriae by testing for mannose sensitivity using a suspension of *Saccharomycetes cerevisiae* [7]. For long-term storage, 8.5 ml of an overnight culture was mixed with 1.5 ml of sterile glycerol and stored at −20°C [8].

Epithelial cells were obtained from one healthy female volunteer with no history of urinary tract infection. Thirty milliliters of freshly voided urine was centrifuged at 4,500 *g* for 10 min to pellet the cells, and the pellet was washed one time with 0.01 M phosphate-buffered saline (PBS), pH 7.4.

Bacterial adherence to exfoliated uroepithelial cells was measured as previously described [1, 10, 11]. One milliliter samples of bacteria containing 10^9 bacteria and 10^5 epithelial cells were combined and incubated in a shaking water bath at 50 oscillations/min for 30 min. Following incubation, a 1 ml sample was removed and filtered through at 8-μm polycarbonate membrane and washed 2 times with 60 ml of deionized water to remove the nonadherent bacteria. The filters were then placed on a glass slide, allowed to dry, and then removed. The adhering cells were gram-stained and a total of 50 epithelial cells were scored for each sample using a light microscope. Statistical comparasions between means were done by analysis of variance (ANOVA). For the incubation studies, 1 ml samples of the bacteria were centrifuged, resuspended in the test medium, and incubated at 37°C for 10 min with shaking. The samples were then mixed with the epithelial cells and subjected to the adherence assay. The test medium included buffer, THP at 8.8, 17.5, 35.0 and 105 μg/ml, urine plus or minus THP, THP at 35.0 μg/ml and calcium chloride at concentrations of 100, 300, 500 and 700 mg/ml. Sodium, potassium and magnesium salts were used at a concentration of 500 mg/l. For each experiment, controls were included in which uroepithelial cells were incubated with and without the addition of *E. coli* without further treatment.

The normal concentration of calcium in the urine ranges from 50 to 250 mg/l [13] and increases to 500 to 660 mg/l after dietary

Table 1. Adherence of *E. coli* to exfoliated uroepithelial cells after incubation in urine plus or minus THP or buffer with increasing concentrations of THP

Treatment	Mean bacteria per cell
Buffer	15.73
Urine	12.27*
Urine minus THP	17.04
THP 105 µg/ml	4.86**
THP 35.0 µg/ml	9.96**
THP 17.5 µg/ml	20.55**
THP 8.8 µg/ml	22.23**

* Significantly different from the buffer control ($P<0.05$)
** Significantly different from the buffer control ($P<0.001$)

Table 2. Adherence of *E. coli* to exfoliated uroepithelial cells after incubation in THP and increasing concentrations of calcium

Treatment	Mean bacteria per cell
Buffer	15.68
THP 35 µg/ml	7.58*
THP 35 µg/ml and 100 mg/l calcium	10.94
THP 35 µg/ml and 300 mg/l calcium	13.38**
THP 35 µg/ml and 500 mg/l calcium	36.36***
THP 35 µg/ml and 700 mg/l calcium	37.50***

* and *** Significantly different from buffer control ($P<0.001$)
** and *** Significantly different from THP minus calcium ($P<0.01$ and 0.001, resepectively)

supplementation with 1,500 mg of calcium carbonate [1]. The normal concentration of THP in the urine ranges from 30 to 50 µg/ml [3, 12].

In a time study adherence of *E. coli* to epithelial cells was examined after preincubation of the bacteria in buffer, THP (35 µg/ml), calcium chloride (500 mg/l) or a combination of THP and calcium chloride at the same concentrations. The preincubation periods were 1, 5, 10, 30, and 120 min. After preincubation, the *E. coli* were mixed with the epithelial cells and subjected to the adherence test described above.

Tamm-Horsfall glycoprotein was prepared from human urine by a slight modification of the method of Tamm and Horsfall [12]. All steps were performed at 4°C. Freshly voided urine was brought to 0.58 M with NaCl and refrigerated for 48 h. The precipitate was collected by centrifugation for 30 min at 4,500 *g*. The supernatant was used immediately for frozen for future use. The pellet was washed 3 times with 50 volumes of 0.58 M CaCl and then resuspended in deionized water and centrifuged one additional time for 30 min at 4,500 *g* to remove any undissolved material. The sample was then dialyzed overnight against two changes of 100 volumes of distilled water. One milliliter of the sample was dried in a vacuum over silica gel to constant weight. The yield ranged from 23 to 28 µg/ml of THP/l or urine.

To determine the homogeneity of the THP preparations, samples were subjected to one-dimensional gel electrophoresis in the presence of 0.1% sodium dodecyl. Since previous determinations [3, 4, 7] estimated the molecular weight of the THP to be between 90 and 100 kD, a 5% polyacrylamide gel was used with a 2.5% stacking gel [2]. The preparations of Tamm-Horsfall protein migrated as a single descreet band having a molecular weight of approximately 90 kD as compared to the protein standards of ovalbumin, 45 kD, bovine serum albumin, 66 kD and B – galactosidase, 116 kD.

Results

Effect of THP on bacterial adherence

Incubation of *E. coli* and epithelial cells in urine resulted in a significant decrease in bacterial adherence as compared to incubation in buffer (Table 1). When urine, from which the THP was removed, was used as the incubation medium, the level of bacterial adherence was not significantly different from the buffer control. Since the urine minus THP also contained 0.58 M NaCl, used in the precipitation step, a separate incubation was carried out in 0.58 M NaCl. The adherence value in 0.58 M NaCl was not significantly different from the value obtained when urine minus THP was used as the incubation medium ($P=0.129$). As an additional control, normal urine containing THP was made 0.58 M with NaCl and used immediately as an incubation medium. When compared to normal urine containing THP, there was no significant difference in adherence ($P=0.889$).

The addition of THP at concentrations of 35 and 105 µg/ml significantly reduced the number of adhering bacteria as compared to the control with no added THP. Concentrations of THP at 17.5 and 8.8 µg/ml significantly increased the number of adhering bacteria as compared to the control value (Table 1).

Effect of calcium on the anti-adherence activity of THP

It can be observed from Table 2 that the addition of THP at a concentration of 35 µg/ml significantly decreased bacterial adherence as compared to the control value. The addition of calcium to the incubation medium at a concentration of 100 mg/l, plus THP, increased the number of adhering bacteria compared to THP alone. The value, however, was not significantly higher. Concentrations of calcium, 300 mg/l or greater, significantly increased the number of adhering bacteria compared to the value with THP alone. Calcium at concentrations of 500 and 700 mg/l plus 35 µg/ml THP increased the number of adhering bacteria to values greater than the control with no added THP.

Time study on the adherence of E. coli to uroepithelial cells after various treatments

E. coli and uroepithelial cells were incubated for 1, 5, 10, 30, and 120 min with various treatments, and the cells were then scored for bacterial adherence (Table 3). For the controls in buffer, adherence values increased to about 30 min and then stabilized. There was no significant difference between incubations of 30 and 120 min ($P=0.297$), whereas incubation periods of 1, 5 and 10 min were significantly different ($P<0.05$) from the 30-min incubation period.

When THP (35 µg/ml) was included in the incubation medium and adherence was monitored at the various time periods, there was no significant difference if the reading at 30 min is compared to 1 ($P=0.396$), 5 ($P=0.608$), 10

Table 3. Adherence of *E. coli* to exfoliated uroepithelial cells after incubation in buffer, THP, calcium, or THP and calcium for increasing periods of time

Time (min)	Treatment/mean bacteria per cell			
	Buffer	THP	Calcium	THP + Calcium
1	20.01*	10.40***	21.90****	21.06******
5	24.55*	10.36***	34.56****	31.05******
10	26.02*	10.12***	44.66*****	37.74*******
30	32.63	12.54	40.86	38.38
120	30.53*******	14.56***	48.21****	47.76******

* Significantly different from 30-min incubation period ($P < 0.05$)
** Not significantly different from 30-min incubation period ($P = 0.297$)
*** Not significantly different from 30-min incubation period; 1 min ($P = 0.396$), 5 min ($P = 0.608$), 10 min ($P = 0.400$), and 120 min ($P = 0.544$)
**** Significantly different from 30-min incubation period ($P < 0.05$)
***** Not significantly different from 30-min incubation period ($P = 0.153$)
****** Significantly different from 30-min incubation period ($P < 0.05$)
******* Not significantly different from 30-min incubation period ($P = 0.618$)

($P = 0.400$), and 120 min ($P = 0.544$). The inclusion of calcium (500 mg/l) in the incubation medium resulted in an increase in adherence at 10 min [30 min is significantly different from 1 and 5 min ($P > 0.05$)], no increase between 10 and 30 min ($P = 0.153$) and an additional increase at 120 min compared to 30 min ($P < 0.05$). When both THP (35 µg/ml) and calcium (500 mg/l) were included in the incubation medium, the pattern was the same as observed for calcium alone; 30 min was significantly different from 1, 5 and 120 min ($P > 0.05$) and not significantly different from 10 min ($P = 0.618$).

Reversal of calcium-induced inhibition of THP anti-adherence activity

An attempt was made to reduce the effect of calcium on THP by successively washing the preparations with distilled water. *E. coli* was incubated with THP and calcium and then subjected to a series of washes prior to incubation with the uroepithelial cells. The results can be observed in Table 4. As in the previous results, THP significantly reduced the number of adhering bacteria compared to the buffer control, and the addition of calcium to the incubation medium reversed this effect. Two washes with distilled water did not significantly reduce the number of adhering bacteria. However, after the third and fourth wash, there was a significant decrease in the number of adhering bacteria compared to incubations including calcium and THP that had not been washed. Also, after four washes the number of adhering bacteria was not significantly different from the incubation in which THP but no calcium was included in the incubation medium.

Table 4. Restoration of THP anti-adherence activity after successive washes with distilled water

Treatment	Mean bacteria per cell
Buffer	20.12
THP 35 µg/ml	13.00*
THP 35 µg/ml and calcium 500 mg/l	32.34**
THP and calcium; wash 1	29.06***
THP and calcium; wash 2	29.34***
THP and calcium; wash 3	17.92****
THP and calcium; wash 4	12.80*****

* Significantly different from control (buffer) ($P < 0.01$)
** Significantly different from THP 35 µg/ml ($P < 0.001$)
*** Not significantly different from THP and calcium with no wash ($P = 0.317$ and 0.374)
**** and ***** Significantly different from THP and calcium with no wash ($P < 0.001$)
***** Not significantly different from THP 35 µg/ml ($P = 0.948$)

Table 5. Effect of additional ions on the anti-adherence activity of THP

Treatment	Mean bacteria per cell
Buffer	32.50
THP 35 µg/ml	22.36*
THP 35 µg/ml + 500 mg/l calcium	44.34**
THP 35 µg/ml + 500 mg/l sodium	27,50***
THP 35 µg/ml + 500 mg/l potassium	26.80***
THP 35 µg/ml + 500 mg/l magnesium	23.32***

* Significantly different from the control (buffer) ($P < 0.001$)
** Significantly different from control and THP 35 µg/ml ($P < 0.001$)
*** Not significantly different from THP 35 µg/ml ($P = 0.074, 0.123$ and 0.750, respectively)

Effect of ions other than calcium on the anti-adherence activity of THP

Chlorine salts of sodium, potassium and magnesium (500 mg/l) were also examined for their effect on the anti-adherence activity of THP (Table 5). Only when calcium chloride (500 mg/l) was included in the incubation medium was there a significant increase in adherence ($P < 0.001$). There was no significant change in adherence when chlorine salts of sodium, potassium and magnesium ($P = 0.074$, 0.123, and 0.750, respectively) were included in the incubation medium.

Discussion

It has been prosposed that Tamm-Horsfall glycoprotein acts in the bladder to trap urinary pathogens containing type-1 fimbriae [9] and may also promote or prevent adherence of these pathogens, depending upon the concentration [3, 4]. We have confirmed that THP may either

promote or prevent adherence *E. coli* to uroepithelial cells, depending upon the concentration. At concentrations of about 35 μg/ml and above, THP acts as an anti-adherence factor while at concentrations of about 17 μg/ml and below, the THP acts to promote bacterial attachment. Normal concentrations of THP in the urine range from about 30 to 50 μg/ml [3, 5, 13]. Thus, it would appear that there is sufficient THP in the urine to inhibit attachment of bacterial pathogens containing type-1 fimbriae. We were able to confirm this suggestion by demonstrating that normal urine significantly reduces bacterial adherence to uroepithelial cells compared to urine from which the THP had been precipitated.

In a previous publication we demonstrated that excess calcium in the urine acts to increase bacterial aderence [1]. In the present investigation we examined the effect of calcium on the anti-adherence activity of THP. At a concentration of 300 mg/l and above, there was a significant decrease in the anti-adherent activity of THP. This is higher than the normal concentration of calcium in the urine which ranges from about 50 to 250 mg/l for a 24-h sample [13]. Thus, only concentrations of calcium higher than those normally occurring in the urine will effectively inhibit the anti-adherence activity of THP. This would suggest that individuals with higher than normal concentrations of calcium in the urine would tend to lose the anti-adherence protection that is normally afforded by THP. Other cations including sodium, potassium and magnesium, even at concentrations almost twice the effective concentration of calcium, had no significant effect on the anti-adherence activity of the THP.

Since it became apparent that high concentrations of calcium in the urine had a detrimental effect on the activity of THP, we wanted to determine how quickly this effect could be observed. Normally we incubated the bacteria and uroepithelial cells for 30 min to allow adherence to occur. In buffer, adherence stabilizes after 30 min. When THP was included in the incubation medium, maximum activity was observed at 1 min with no significant change in adherence up to 120 min. Thus, while it takes about 30 min for maximum adherence to occur, the protective activity of THP is available almost immediately. The inclusion of calcium in the incubation medium resulted in a progressive increase in adherence up to the maximum time period of 120 min, and THP with calcium showed the same pattern. Therefore, calcium is a very strong promoter of bacterial adherence and quickly negates the anti-adherence effect of THP.

To determine how strongly the calcium was associated with the THP on the bacterial cells, bacteria incubated with THP and calcium were subjected to a series of washes. It was found that after three washes there was a significant decrease in bacterial adherence, and after the fourth wash the adherence value was the same as the THP control. Thus, the association of calcium with the THP on the bacterial cell surface seems to be transient. We previously demonstrated the calcium could be washed from the surface of both bacterial and epithelial cells with a concurrent decrease in bacterial adherence. In a previous publication we demonstrated that washing the bacteria four times had no effect on bacterial adherence [1]. In the current investigation it was observed that after four washes the anti-adherent activity of THP was still evident, indicating the washing process did not remove THP. A similar observation has been made by Duncan [4].

In conclusion, THP can act as an anti-adherence agent at concentrations that occur in normal urine and thus may act as a protective agent for urinary tract infections. THP activity is reversed by calcium at concentrations higher than normally occurring in the urine. Therefore, individuals with increased concentrations of calcium in the urine could be at increased risk for urinary tract infections. This may be particularly significant for individuals who are using calcium supplements. THP activity is retained after washing the cells with an aqueous solution whereas calcium activity is lost. This suggests that as the level of calcium is reduced in the urine, the association of THP with calcium is reduced, thus restoring the anti-adherence activity of THP.

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