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Neutrophil function in hyperosmotic NaCl is preserved by phosphoenol pyruvate

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Summary. Hyperosmolority in the urinary tract inhibits the host defense against bacterial infection. NaCl contributes most to osmolority in the renal medulla and urine. Therefore, we studied the effect of hyperosmolar NaCl on neutrophil function. When osmolarity was increased by NaCl, polymorphonuclear leukocytes (PMNL) became defective in phagocytosis, intracellular killing of bacteria, chemotactic activity, and superoxide production. This coincided with a decrease in the intracellular adenosine triphosphate (ATP) content of PMNL. Both the inhibition of superoxide production and the reduction in ATP content did not occur at 4°C. By increasing ATP content, phosphoenol pyruvic acid (PEP) protected against the decrease in superoxide production. These results suggest that leukocyte function is inhibited by high concentrations of NaCl due to the activation of the Na^+-K^+ pump. PEP, an ATP precursor, can protect PMNL against osmotic stress by raising the intracellular concentration of ATP.

Key words: Hyperosmolarity – NaCl – Neutrophil – Phosphoenol pyruvic acid – ATP

High osmolarity is physiologic in the urine and renal medulla, which are the sites of host-parasite interaction in urinary tract infections. NaCl and urea contribute most to the hyperosmolarity in the urinary tract. We reported previously that some leukocyte functions were suppressed by hyperosmolarity due to NaCl and urea [8]. The suppression by NaCl was due to energy depletion, and the suppression by urea was due to nonspecific inactivation of polymorphonuclear leukocytes (PMNL) [9]. At that time, we were not aware of any substance which could preserve leukocyte function under hyperosmolar stress [2]. We examined precursors of adenosine triphosphate (ATP) such as adenine, inosine, or phosphoenol pyruvic acid (PEP) for protection of PMNL against NaCl hyperosmolarity. We report here that PEP is a potent protectant of PMNL against hyperosmolar NaCl.

Materials and methods

Polymorphonuclear leukocytes

PMNL were obtained from the peripheral blood of healthy volunteers after informed consent. Blood was collected on 3% citric acid and allowed to sediment in dextran for 1 h. Leukocyte-rich supernatant was separated by centrifugation on Histopaque (Sigma). After lysis of residual erythrocytes by hypotonic shock, the cells were resuspended in Hank's balanced salt solution (HBSS) (GIBCO) and stored on ice until use. Each experiment was performed in triplicate.

Osmotic substance

NaCl was used to modify the osmolarity of HBSS (140 m*M* NaCl), the suspension medium for all experiments. The NaCl concentration was varied according to the aims of the experiments.

Phagocytosis and intracellular killing of bacteria by PMNL

Phagocytosis of bacteria was measured by the method of Leijh et al. [5]. Briefly, a suspension of 10^7 PMNL/ml was incubated with an equal volume of 10^7 cfu/ml of bacteria with 10% (v/v) fresh homologous serum at 37°C. To stop phagocytosis at various times, a 0.5-ml sample of the suspension was added to 1.5 ml ice-cold HBSS. After centrifugation at 110 *g*, the number of viable bacteria in the supernatant was determined by the microbiological plate method. Phagocytosis at any given time is expressed as the percent decrease in the initial number of viable extracellular bacteria. Intracellular killing of bacteria was also measured by the method of Leijh et al. [6]. Briefly, a suspension of 5×10^6 PMNL/ml and 5×10^6 preopsonized bacteria per milliliter was incubated at 37°C, after which phagocytosis was stopped by shaking the tubes in ice-cold water. The extracellular bacteria was removed by centrifuging the suspension for 4 min at 100 *g* and washing the cells twice with ice-cold HBSS. The cells containing ingested bacteria were then resuspended in HBSS to a concentration of 5×10^6 PMNL/ml and reincubated at 37°C. After various periods of reincubation, the intracellular killing was stopped by adding 0.5 ml of ice-cold HBSS to 0.5 ml of the cell suspension. The cells were then spun down for 4 min at 110 *g*, the supernatant removed, and the cells lysed by adding 10 ml of distilled water. Then, the number of viable bacteria was determined. The

Table. 1. Osmolality of solutions and viability of PMNL which were incubated with various concentrations of NaCl for 30 min

Test solution	Concentration (mM)	Osmolality (mosmol/kg)	Viability[a] (%)
NaCl	140	255	99.0
	270	515	98.0
	370	715	88.0
	470	930	81.7

(PMNL, polymorphonuclear leukocytes
[a] Viability was determined by the trypan blue dye exclusion test. Each value is the mean of 3 different experiments

Table 2. Inhibition of chemotactic activity of PMNL stimulated by opsonized zymosan and FMLP in various concentrations of NaCl

Chemoattractant	Concentration of NaCl (m*M*)	No. of PMNL/HPF (Mean ± SD; $n=5$)	% Control	*t*-Test
Opsonized zymosan (5%)	140	266 ± 58	100 ± 22	]*]*]*
	270	120 ± 31	45 ± 22	
	370	2 ± 2	1 ± 1	
	470	4 ± 4	2 ± 2	
FMLP ($10^{-7}M$)	140	217 ± 36	100 ± 17	]*]*]*
	270	3 ± 3	1 ± 1	
	370	1 ± 1	0 ± 0	
	470	0 ± 0	0 ± 0	

FMLP, Formyl-methionyl-leucyl-phenylalanine; PMNL, polymorphonuclear leukocytes; HPF, high power field; * $P<0.001$

extent of intracellular killing at a given time is expressed as the percent decrease in the initial number of viable intracellular bacteria. All experiments were performed in triplicate.

Chemotaxis of PMNL

Chemotaxis was measured by the modified Boyden's method [11]. The blindwell chemotaxis chamber (BIO-PAD) was used with a 3-nm pore polycarbonate filter; 2×10^7 PMNL were placed in the upper well. Formyl-methionyl-leucyl-phenylalanine (FMLP) at 10^{-7} *M* or 5% serum-activated zymosan was placed in the lower well as a chemoattractant. After incubating the chambers for 1 h at 37°C and staining with Giemsa, the PMNL penetrating the filter were counted. This number was represented as the mean numbers of PMNL in each of five high power fields. Osmotic solutions were added to both upper and lower wells.

Superoxide production:

Superoxide production was examined according to Babior and Cohen [1] using phorbol myristate acetate (PMA; Sigma) as a stimulus and horse heart cytochrome C, Type III (Sigma), as a substrate. Two kinds of fixed time assays, the tube and the microplate methods, were used. PMNL were suspended at a concentration of 1.5×10^6 cells/ml in HBSS and preincubated for 30 min with or without the test solution at 37°C in a shaking water bath. Then, superoxide production was determined using 3 μg of PMA and 30 mg/l of cytochrome C. Incubation for superoxide production was carried out for 30 min at 37°C. The OD was measured at 550 nm in a spectrophotometer. Superoxide production (nmol/10^6 PMNL per minute) was calculated according to the formula:

$$\text{Superoxide production} = \frac{(\text{OD test} - \text{OD blank}) \times \text{volume} \times 47.4}{\text{incubation time}}$$

Results were expressed as percent control activity in PMNL incubated in plain HBSS for similar periods of time. All experiments were performed in triplicate.

Determination of intracellular ATP content

The intracellular ATP content was measured by the Luciferin-Luciferase method. Briefly, 100 μl of a PMNL suspension (1×10^6 cells/ml) was mixed with an ATP-releasing reagent for 60 s. Then 0.1 ml of the Luciferin-Luciferase mixture was added and the light production measured using a TD 4000 lumiphotometer (Labo Science). The intracellular ATP content was calculated using an internal ATP standard (10 μl of 3×10^{-7} mol/ml). All experiments were performed in triplicate.

ATP precursor

The ATP precursors, adenine, inosine, and PEP, were studied. A solution of 5 m*M* adenine hydrochloride, 10 m*M* inosine or a solution containing both were added to the NaCl solution in order to increase the intracellular ATP content. PEP was also used at concentration of 10 m*M*.

Statistical analysis

The difference between experimental groups was considered significant, $P<0.05$, according to the Student's *t*-test.

Results

Osmolality of solutions and cell viability

Unaltered HBSS is 140 m*M* with respect to NaCl and contains 255 mosmol/kg. HBSS solutions modified to contain 270, 370, and 470 m*M* of NaCl had osmolalities of 515, 715, and 930 mosmol/kg, respectively. The viabilities of PMNL incubated in these solutions for 30 min ranged from 81.7% to 98.0% (Table 1).

Suppression of phagocytosis and bactericidal activity

Phagocytosis of bacteria by PMNL in the solutions containing 270 and 370 m*M* of NaCl was reduced when compared to the solution containing 140 m*M* NaCl. Intracelluler bactericidal activity was suppressed completely in hyperosmotic solutions of NaCl (Fig. 1).

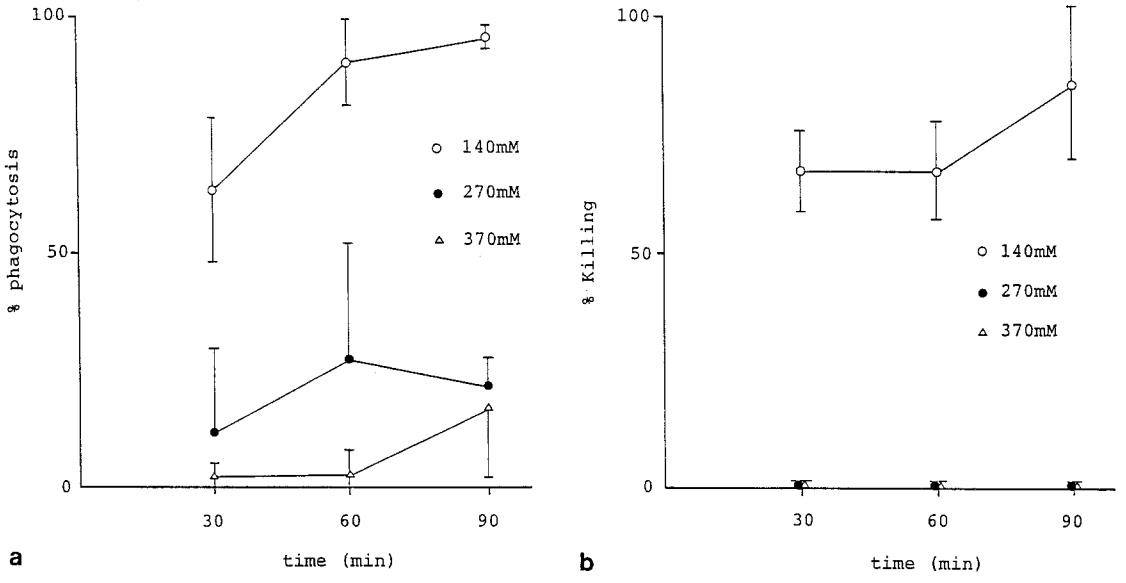

Fig. 1 a, b. Percent phagocytosis **(a)** and killing **(b)** of *Escherichia coli* (ATCC 25922) by polymorphonuclear leukocytes (PMNL) incubated with various concentrations of NaCl for 30, 60, and 90 min. Each value is the mean of three experiments ± SD

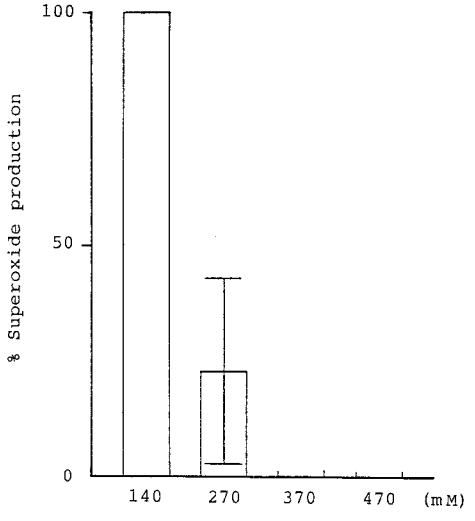

Fig. 2. Percent superoxide production in hyperosmolar NaCl by polymorphonuclear leukocytes (PMNL) stimulated with phorbol myristate acetate (PMA) compared to that in the normosmolar solution. Each value is the mean of three experiments ± SD

Suppression of chemotaxis

Chemotaxis was also suppressed by hyperosmolar NaCl. The number of PMNL penetrating the membrane was small under hyperosmolar conditions as compared to controls, even when PMNL were stimulated by both opsonized zymosan and FMLP (Table 2).

Suppression of superoxide production

Superoxide production by PMNL stimulated with PMA was also suppressed when PMNL were preincubated in hyperosmolar solutions in proportion to the concentration of NaCl (Fig. 2).

Reduced intracellular ATP content

To determine whether changes in intracelular energy stores could mediate the dysfunction seen in PMNL incubated in hyperosmolar NaCl, we determined the intracellular ATP content of PMNL. In both 270 and 370 m*M* NaCl, the intracellular ATP content was reduced when compared to that in 140 m*M* NaCl (Fig. 3).

Temperature dependence of suppressing superoxide production and reducing intracellular ATP content

Superoxide production by PMNL was suppressed when PMNL were preincubated with hyperosmolar NaCl at

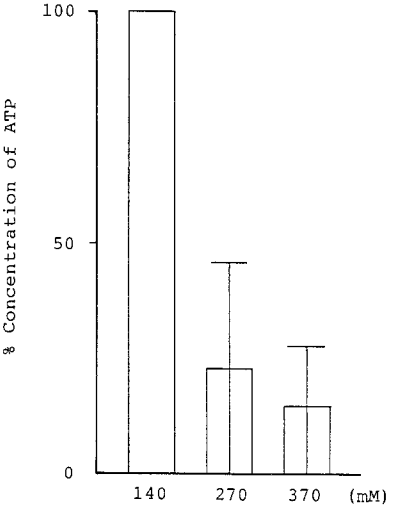

Fig. 3. Percent concentration of ATP in polymorphonuclear leukocytes (PMNL) incubated in hyperosmolar NaCl for 30 min. Each value is the mean of three experiments ± SD

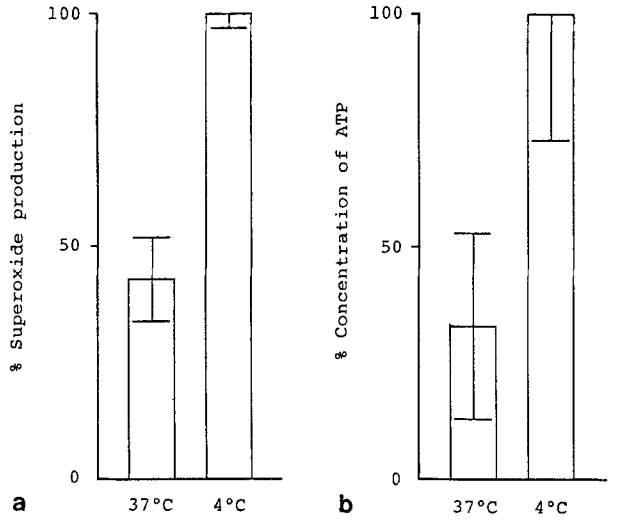

Fig. 4a, b. Temperature during incubation. Percent superoxide production **(a)** and intracellular ATP **(b)** concentration in polymorphonuclear leukocytes (PMNL) preincubated with moderately hyperosmolar NaCl solution (270 m*M*) at 37°C or 4°C. Each value is the mean of three experiments ± SD

Table 3. Superoxide production by PMNL preincubated in NaCl solution (370 m*M*) with or without adenine or inosine

Solution	% Superoxide production (Mean ± SD; n = 3)	
HBSS	100 ± 0	NS
HBSS + adenine hydrochloride (10 m*M*)	108 ± 12	
HBSS + inosine (5 m*M*)	100 ± 11	
HBSS + adenine (10 m*M*) + inosine (5 m*M*)	105 ± 11	
NaCl (370 m*M*)	68 ± 12	NS
NaCl (370 m*M*) + adenine hydrochloride (10 m*M*)	67 ± 20	
NaCl (370 m*M*) + inosine (5 m*M*)	70 ± 20	
NaCl (370 m*M*) + adenine + inosine (5 m*M*)	74 ± 28	

PMNL, polymorphonuclear leukocytes; NS, not significant

37°C for 30 min as described above. However, when PMNL were preincubated with hyperosmolar NaCl at 4°C, superoxide production was not suppressed at all. This correlated well with the reduction in intracellular ATP content seen after preincubating PMNL with hyperosmolar NaCl at 37°C and the lack of change in intracellular ATP content after preincubating with hyperosmolar NaCl at 4°C (Fig. 4).

Effect of adenine or inosine on superoxide production by PMNL subjected to hyperosmolar NaCl

When PMNL were preincubated in hyperosmolar NaCl for 30 min, superoxide production did not increase after adding inosine, adenine hydrochloride, or an adenine-inosine mixture (Table 3).

Effect of PEP on superoxide production by PMNL treated with hyperosmolar NaCl

PEP preserved superoxide production by incubating PMNL in a hyperosmolar NaCl (370 m*M*) solution. This protective effect was seen for treatments of 30 and 45 min in duration (Table 4).

ATP content of PMNL preincubated in NaCl solution with or without PEP

The intracellular ATP content was measured after PMNL were treated with HBSS, NaCl solution, and NaCl solution each containing 10 m*M* PEP. The concentration of ATP was lower in PMNL treated with hyperosmolar NaCl. However, when PMNL were treated with NaCl solution containing PEP, the ATP content actually increased (Fig. 5).

Discussion

Mammalian cells regulate their volume and intracellular ion distribution. The main mechanism regulating intracel-

Table 4. Phosphoenol pyruvic acid preserves superoxide production by PMNL subjected to hyperosmolar NaCl (370 m*M*)

Incubation time	Solution	% Superoxide production					*t*-Test
		Exp 1	Exp 2	Exp 3	Exp 4	Mean ± SD	
30 min	HBSS	100	100	100	nd*	100 ± 0	ns
	HBSS + PEP	72.8	83.7	106	nd	87.4 ± 17.1	
	NaCl	36.2	46.0	36.5	nd	39.6 ± 5.6	$P < 0.05$
	NaCl + PEP	50.9	47.7	50.4	nd	49.7 ± 1.7	
45 min	HBSS	100	100	100	100	100 ± 0	
	NaCl	37.4	48.1	39.3	44.0	42.2 ± 4.8	$P < 0.05$
	NaCl + PEP	46.7	64.2	48.0	49.8	52.2 ± 8.1	

nd, Not done; ns, not significant; PMNL, polymorphonuclear leukocytes; Exp, experiment; PEP; phosphoenol pyruvic acid

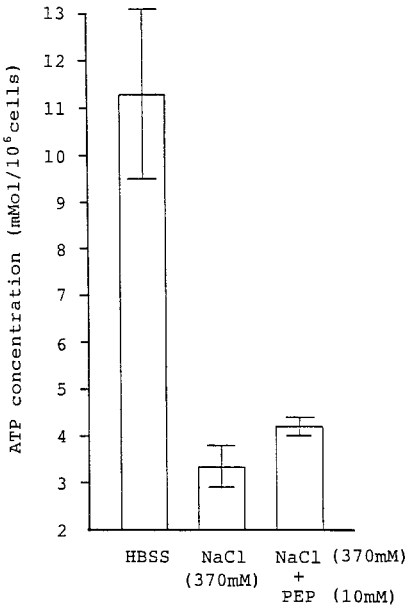

Fig. 5. Intracellular ATP content of polymorphonuclear leukocytes (PMNL) preincubated in normosmolar solution (HBSS), hyperosmolar NaCl (370 m*M*), and hyperosmolar NaCl with 10 m*M* phosphoenol pyruvic acid (PEP) for 30 min. Each value is the mean of three experiments ± SD

lular osmolarity during extracellular osmotic stress is thought to be the Na^+-K^+ pump mediated by Na^+-K^+ ATPase [7]. However, this regulatory mechanism is not well defined in PMNL subjected to hyperosmotic stress. We suggested previously that the mechanisms protecting PMNL against hyperosmotic stress are different for NaCl than for urea osmoles. Inhibition of PMNL by NaCl is energy-dependent, presumably because the cell possesses a compensatory mechanism. Urea, however, seems to inhibit PMNL nonspecifically. Furthermore, inhibition of PMNL function and depletion of intracellular ATP correlates in the case of hyperosmolar NaCl. These findings suggest that inhibition of PMNL by hyperosmolar NaCl occurs because the compensating Na^+-K^+ pump depletes intracellular ATP, diverting the energy pool used normally by PMNL fo other cellular functions [9].

In this study, we demonstrated that hyperosmolar NaCl suppressed many PMNL functions, such as phagocytosis, intracellular bactericidal activity, chemotaxis, and superoxide production, and that this suppression is due to intracellular ATP depletion.

In general, stored erythrocytes have a limited ability to transport oxygen because of decreased ATP or ATP precursors. Blood is stored commonly in acid-citrate-dextrose (ACD) solution. However, the intracellular ATP content is low in erythrocytes stored in ACD solution at 4° C for a few weeks. The addition of adenine and inosine to the ACD solution is reported to be effective for the maintenance of intracellular ATP in stored erythrocytes [10].

Our studies demonstrate that adenine and inosine were not effective in maintaining the function of leukocytes subjected to hyperosmolar NaCl. Recently, it was reported that PEP permeated readily the erythrocyte membrane and increased intracellular ATP and 2,3-bis-phosphoglycerate (2,3-BPG) content. Stored erythrocytes can be revived by increasing ATP and 2,3-BPG concentration by 60 min of incubation in ACD solution supplemented with PEP. Similarly, we found that in PMNL subjected to hyperosmolar NaCl, superoxide production and intracellular ATP content was maintained by PEP.

In conclusion, PEP is thought to protect PMNL in hyperosmolar NaCl by maintaining intracellular ATP. Thus, PMNL dysfunction in the urinary tract may in part be due to intracellular ATP depletion as PMNL activate their Na^+-K^+ pump to maintain intracellular osmolarity.

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