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Effect of NaCl and Urea Concentration Comparable to Renal Medulla on Superoxide Production by Human Polymorphonuclear Leukocytes

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Summary. The influence of hyperosmolarity on superoxide production by polymorphonuclear leukocytes (PMNL) was examined using NaCl and urea as osmotic substances. Superoxide production was inhibited in a hyperosmotic environment produced by high concentrations of these substances with the following IC_{50}: 440 ± 75 (SD) mOsm/kg for NaCl and 660 ± 100 for urea. In the case of NaCl, this inhibition was time-dependent and abolished at 4 °C. Since PMNL pump out Na^+ ion for maintenance of cellular volume in an energy dependent fashion, it was suggested that the inhibition of superoxide production was due to the exhaustion of energy stores. On the other hand, urea inhibition was almost immediate and remained even when preincubation was performed at 4 °C. Because the transport of urea through the cell membrane is known to be energy independent, these findings suggested that urea was either an inhibitor of the NADPH oxidase or a scavenger of superoxide anion.

Key words: Superoxide – PMNL – Hyperosmolarity – NaCl – Urea – Pyelonephritis

Introduction

Polymorphonuclear leukocytes (PMNL) are one of the most effective defense-mechanisms against renal bacterial infection. It has been reported that the renal medulla is especially susceptible to bacterial infection due to the adverse effect of the hyperosmotic environment of the renal medulla on PMNL [7]. Chernew and Braude [5] and Lancaster and Allison [9] reported that the phagocytic function of PMNL was suppressed in hyperosmotic conditions caused by high concentration of NaCl or urea. Bryant et al. [4] also reported that functions such as migration, aggregation, adhesion, phagocytosis and intracellular killing of bacteria were suppressed by high concentration of NaCl, urea and sucrose. They showed that leukocyte functions were suppressed in high osmotic conditions comparable to those of the renal medulla. However, the mechanism of the inhibition is not yet clear. Furthermore, the influence of high osmolarity on superoxide production by PMNL has not yet been studied.

It has been reported that the main cause of renal scarring during pyelonephritis is the presence and activation of PMNL [3, 8, 14]. Oxidative mechanisms of PMNL may play a significant role in this regard [12]. Therefore, we studied the influence of NaCl and urea concentration on superoxide production by PMNL and report here that the superoxide production of PMNL is suppressed by hyperosmolarity generated by NaCl, urea and polyethylene glycol (PEG) and discuss the mechanism of this suppression.

Materials and Methods

Neutrophils

Neutrophils (PMNL) were obtained from the peripheral blood of healthy volunteers who had given informed consent. Blood (20 to 60 ml) was collected on ACD. Following sedimentation in dextran for 1 h was allowed, the leukocyte-rich supernatant was separated on Ficoll-Hypaque (Pharmacia). After lysis of the residual erythrocytes by hypotonic shock, cells were resuspended in HBSS (Gibco) and stored on ice until use. Counting and viability tests were done before each experiment. Viability was measured by trypan blue dye exclusion test. The final preparation was >97% PMNL and >95% viable.

Effect of NaCl, Urea and PEG

NaCl, urea and polyethylene glycol (PEG 200, MW: 190–200, Merck, Darmstadt, FRG) were used as osmotic substances, because NaCl and urea are the main substances responsable for hyperosmolarity in the renal medulla and PEG was used as a pure osmotic substance with low-toxicity to the PMNL.

Variation in osmolarity (255–1,120 mOsm/kg) was obtained by various concentration of NaCl, urea and PEG. Hanks' balanced

salt solution (HBSS, Gibco) was used as the suspension medium for all experiments, which contains 140 mM of NaCl, and the solutes were altered according to the aims of the experiments. Osmolarity was measured using a Halbmikro-osmometer (Knauer, Vel, Belgium).

The effect to temperature was studied by preincubating the PMNL at 4 °C instead of at 37 °C. Recovery of the toxic effects of hyperosmolarity was studied by washing the preincubated PMNL and resuspending them in HBSS with or without glucose, for various periods of time before the superoxide production test.

Superoxide Production

Superoxide production was studied according to Babior and Cohen [2] using phorbol myristate acetate (PMA) (Sigma) as the stimulus and horse heart cytochrome C (Sigma, type III) as the substrate. Both a fixed time and a continuous assay 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 test solution at 37 °C in a shaking water-bath, and then, superoxide production was determined by fixed time assay, using 3 μg of PMA and 30 mg/ml of cytochrome C. Incubation for superoxide production was carried out for 30 min at 37 °C. The OD was measured at 550 nm. The amount of superoxide production (nmol/10^6 PMNL/min) was calculated from the formula: (OD test-OD blank) x volume of incubation mixture x 47.4 divided by the incubation time. Results were expressed as a percentage of the control activity obtained with PMNL incubated in HBSS for a similar period of time. For the continuous assay, 5×10^5 PMNL were in HBSS mixed with PMA and cytochrome C, OD was recorded automatically for 10 min and superoxide production was calculated from the slope of the graph. Results were also expressed as percentages of the control activity.

Results

Superoxide Production by PMNL preincubated with Various Concentrations of NaCl, Urea or PEG

When PMNL were incubated with the hyperosmotic solutions, superoxide production was dose-dependently inhibited. Table 1 shows the inhibition of superoxide production, as measured by fixed time assay, by high concentration on NaCl, urea or PEG, in which PMNL were incubated for 30 min. The inhibition of superoxide production of PMNL were concentration-related, and particularly was related to the osmolarity of these solutions. The IC50 (50% inhibition concentration; mean ± SD) were 440 ± 75, 660 ± 100 and 730 ± 80 mOsm/kg for NaCl, urea and PEG, respectively, as shown in Fig. 1. Viability of PMNL was marginally affected by NaCl but neither by urea nor by PEG after 30 min of incubation.

Time Course Study

PMNL were incubated with 370 mM of NaCl (715 mOsm/kg) or 400 mM of urea (690 mOsm/kg) solution for 5, 10 and 30 min. The inhibition by NaCl required at least 10 min of preincubation whereas urea inhibited superoxide production more rapidly (Fig. 2).

Table 1. Superoxide production by PMNL preincubated with NaCl, urea or PEG for 30 min (fixed time assay)

Solution	Concentration (mM)	Osmolarity (mOsm/kg)	No. of tests	Viability (%)	% Superoxide production (mean ± SD)
NaCl	140	255	3	99.0	100 ± 0
	270	515	3	98.0	23.8 ± 20.1
	370	715	3	88.0	0 ± 0
	470	930	3	81.7	0 ± 0
Urea	0	255	4	99.3	100 ± 0
	10	305	3	96.0	96.5 ± 3.5
	100	390	3	97.7	92.9 ± 1.9
	200	490	4	97.7	82.7 ± 25.8
	400	690	4	96.7	44.8 ± 38.5
	600	910	3	94.0	0.1 ± 0.1
	800	1,120	3	96.0	0 ± 0
PEG[a]	0	255	3	97.0	100 ± 0
	–	289	3	96.0	92.5 ± 9.3
	–	387	3	98.0	78.8 ± 26.5
	–	431	3	98.4	74.7 ± 32.3
	–	657	3	96.7	60.0 ± 21.4
	–	887	3	96.0	21.0 ± 2.6
	–	960	3	96.0	0 ± 0

[a] PEG = Polyethylene glycol 200

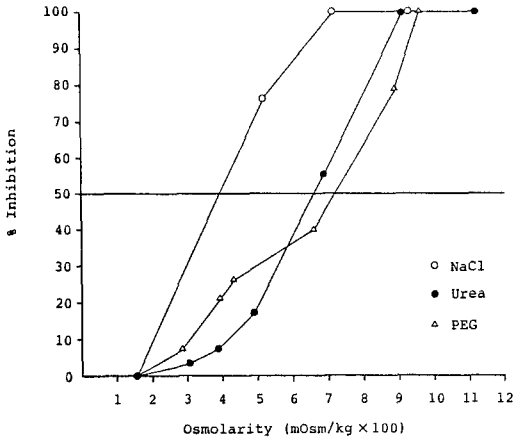

Fig. 1. Percent inhibition of superoxide production by PMNL preincubated with various osmotic solutions of NaCl (○), urea (●) and PEG (△). PMNL were preincubated for 30 min with osmotic solutions and superoxide production was measured by fixed time assay. Each result is the mean of 3 experiments

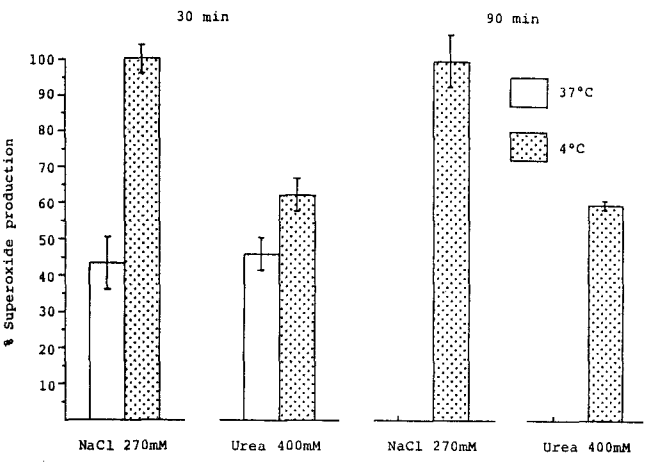

Fig. 3. Superoxide production by PMNL preincubated with NaCl (270 mM) or urea (400 mM) solution for 30 or 90 min at 37 °C (□) or 4 °C (▨). Continuous assay was used for measurement of superoxide production

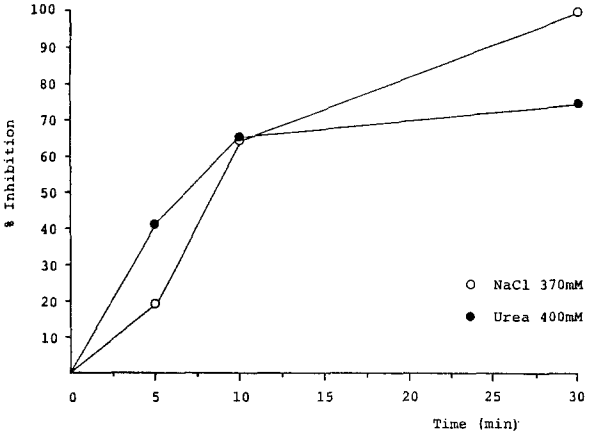

Fig. 2. Time course study of the inhibition of superoxide production by PMNL, which were preincubated with NaCl (370 mM) solution (○) or urea (400 mM) solution (●). Each result is the mean of 2 experiments

Temperature Dependency of the Inhibition of Superoxide Production

When PMNL were preincubated with NaCl (270 mM) at 37 °C for 30 and 90 min, the corresponding superoxide production was 43.2% and 0%. In contrast, when PMNL were preincubated at 4 °C, superoxide production was not inhibited at all. In the case of urea, superoxide production was similarly inhibited both at 37 and 4 °C after 30 min of preincubation. When PMNL were preincubated for 90 min with urea solution, superoxide production was 0% and 59.6% at 37 and 4 °C, respectively (Fig. 3). In the case of PEG, temperature dependency of the inhibition of superoxide production was similar to NaCl (Data not shown).

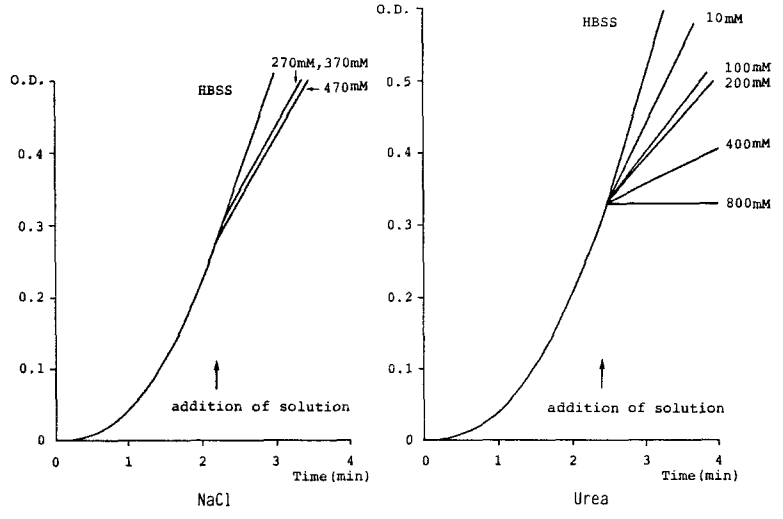

Fig. 4. Continuous assay of superoxide production by PMNL, which was started for assay and various kinds of NaCl or urea solution were added in the cuvette during the assay

Table 2. Absence of recovery of the inhibition of superoxide production by PMNL preincubated with NaCl or urea solution. PMNL were preincubated with NaCl (370 mM) or urea (400 mM) solution for 10 min, washed twice with HBSS and incubated with HBSS for 0, 15, 30, 60 or 90 min

Preincubation solution	Time of incubation with HBSS (min)	Superoxide production (%)		a/b
		Preincubated with		
		solution[a]	HBSS[b]	
NaCl (370 mM)	0	67.2	100.0	67.2
	15	18.9	49.6	38.1
	30	11.3	42.0	26.9
	60	9.7	45.4	21.4
	90	6.3	25.2	25.0
Urea (400 mM)	0	67.9	100.0	67.9
	15	8.7	78.6	11.1
	30	13.4	67.9	19.7
	60	10.0	73.3	13.6
	90	2.0	21.9	9.1

[a] PMNL were preincubated with NaCl or urea, washed and incubated with HBSS
[b] PMNL were preincubated, washed and incubated with HBSS

Table 3. Absence of recovery of superoxide production by the addition of 1 or 2% glucose. Preincubation of PMNL was performed for 10 min with preincubation-mixture, and the superoxide production was measured by fixed time assay

Preincubation mixture	Osmolarity (mOsm/kg)	Superoxide production (% of control)
HBSS	255	100
HBSS + 1% glucose	295	84.8
HBSS + 2% glucose	355	42.8
NaCl (270 mM)	475	85.0
NaCl + 1% glucose	515	52.2
NaCl + 2% glucose	570	17.6
NaCl (370 mM)	655	46.2
NaCl + 1% glucose	695	3.1
NaCl + 2% glucose	740	0.0

Table 4. Absence of recovery of superoxide production by the addition of 1% glucose. PMNL were preincubated with HBSS or NaCl solution with or without glucose for 10 min, followed by 30 min of incubation with or without glucose. Superoxide production was measured by continuous assay

Preincubation mixture	Incubation mixture	Superoxide production (% of control)
HBSS	HBSS	100
HBSS	HBSS + 1% glucose	132
HBSS + 1% glucose	HBSS	106
HBSS + 1% glucose	HBSS + 1% glucose	76.0
NaCl (370 mM)	HBSS	2.9
NaCl	HBSS + 1% glucose	0
NaCl + 1% glucose	HBSS + 1% glucose	4.4
NaCl + 1% glucose	NaCl + 1% glucose	0

Direct or Indirect Inhibition

In order to study whether the inhibition of superoxide production by NaCl and urea was due to direct or indirect interaction with superoxide generation activity of PMNL, various concentration of NaCl or urea solution were added and superoxide production was measured by continuous assay. When 270, 370 or 470 mM of NaCl solution were added, superoxide production was minimally influenced during the first minutes. In contrast, when various concentrations of urea were added, superoxide production was immediately inhibited, with a concentration-dependent relationship (Fig. 4).

Recovery Experiment of the Inhibition of Superoxide Production

When PMNL were preincubated with NaCl (370 mM) or urea (400 mM) for only 10 min, washed twice with HBSS, and then incubated with HBSS only for 15, 30, 60 or 90 min, the inhibition of superoxide production was not restored in each case (Table 2).

Similarly, the inhibition of superoxide production by 270 mM or 370 mM of NaCl solution was not restored by the addition of 1 and 2% glucose at preincubation (Table 3) even after correction for the contribution of glucose to the final osmolarity. Furthermore, when PMNL were prein-

cubated with HBSS or NaCl with or without glucose, followed by 30 min of incubation with HBSS containing 1% glucose, superoxide production was not restored (Table 4).

Discussion

All animal cells must regulate their intracellular volume and water content against osmotic stress. It has been known that two regulatory mechanisms, one ouabain-sensitive and one ouabain-resistant, mediate the cellular volume and intracellular ion distribution. The ouabain-sensitive mechanism is dependent on Na^+-K^+ pomp mediated by Na^+-K^+ ATPase [10]. These regulatory mechanisms have widely been studied in erythrocytes, lymphocytes and granulocytes against hypoosmotic stress only [1, 6]. Few investigations about the influence of hyperosmolarity against cellular volume and function have been performed except for renal papillary cells [11].

Some investigators reported leukocyte function in hyperosmotic condition [4, 5, 13] and showed that many functions such as phagocytosis, aggregation, chemotaxis and intracellular killing of bacteria were suppressed by hyperosmolarity generated by high concentrations of NaCl, urea or some sugars. The mechanism of this suppression, however, remains unclear.

We showed that hyperosmolarity, produced by high concentration of NaCl, urea or PEG, inhibited superoxide production by PMNL, and that this inhibition was different for NaCl and urea. First, the inhibition of superoxide production by NaCl was higher than that by urea, at corresponding osmolarity. Second, the inhibition occurred more rapidly in urea than in NaCl. Third, the inhibition by NaCl but not by urea was presented when PMNL were stressed at 4 °C. Finally, superoxid production was inhibited directly and rapidly by urea in contrast to NaCl, as assessed by a continuous assay. These findings suggest that the inhibition of superoxide production of PMNL by NaCl was indirect and metabolism- or energy-dependent, and the inhibition by urea was direct and metabolism- or energy-independent.

In general, animal cells require much energy for regulation and maintenance of cellular volume and intracellular ion distribution. In particular, one third of the cell energy is consumed in fueling the Na-K pomp. Much more energy may be required at high NaCl concentration. Therefore, it was suggested that superoxide production was suppressed by high concentration of NaCl through energy exhaustion.

On the other hand, urea freely diffuse through the membrane of PMNL and its transport is energy-independent. The mechanism of inhibition may be either inhibition of the NADPH oxidase through denaturation of the enzyme or decoupling the flavoprotein and cytochrome C or a scavenger of superoxide anion. The absence of recovery after preincubation in HBSS (Table 2) suggests that urea exerts its inhibitory effect through denaturation of the enzymatic complex.

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