Urea Transport Across Peritoneal Membrane *In Vitro*: Influence of Protamine Sulfate, Glyoxal, and Methylglyoxal

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*Charge factors and reactive carbonyl solutes may change peritoneal structure and the transport properties of peritoneum. The aim of the present study to analyze the influence of polycationic protamine and glucose degradation products on the diffusive permeability (*P*) of peritoneal membrane for urea* in vitro. *Values for diffusion from the interstitial (I) to the mesothelial (M) side of the membrane and in opposite direction are expressed as coefficients of diffusive permeabilit. Four separate series of experiments were conducted. In the first experiment, transperitoneal transfer of urea (20 mg/dL) in control conditions over 120 minutes was analyzed. In the subsequent three experiments, transport parameters were analyzed before (15 – 60 minutes) and after (75 – 120 minutes) the addition of chemical factors (protamine sulfate 5 mg/dL, glyoxal 10 mg/dL, methylglyoxal 1 mg/dL) on the mesothelial side of the peritoneal membrane. Stability of urea transport was observed in the control series (120 minutes). The mean diffusive permeability coefficients (*P *± standard error of the mean) were 2.293 ± 0.211 cm/s and 2.621 ± 0.457 cm/s (×0.0001) for I→M and M→I transfer respectively. Protamine and methylglyoxal did not alter transport, but glyoxal lowered urea M→I transport by 12% (*p *< 0.01), with a statistically nonsignificant reduction in opposite direction. Similar modifications are observed* in vivo *during peritoneal dialysis and may influence the efficiency of renal replacement therapy.*

Key words

Peritoneal transport, urea

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Introduction

Chronic kidney disease is an increasing clinical problem because of rising life expectancies and the accompanying metabolic syndromes and diseases of civilization (1). Over a number of decades, peritoneal dialysis (PD) has become an established, adequate, and well accepted renal replacement therapy. The relatively large anatomic surface area of the semipermeable peritoneum (about 1.75 m^2 in adult humans) is effectively utilized to remove water and uremic toxins from the body fluid of patients. One of the most important challenges in PD is the chronic preservation of peritoneal membrane integrity (1–3).

The peritoneal membrane is a complex negative-charge barrier with several transfer resistances such as endothelium, interstitium, mesothelium, and stagnant fluid layers on the borderline of the tissue surface (1,2,4,5). The membrane's role in permeability to individual solutes remains not extensively defined. Moreover, the functional–structural properties of the barrier can change in parallel with alterations in the conditions of PD therapy—for example, uremic toxins in the body and unphysiologic components in dialysis solutions such as glucose in high concentration, glucose degradation products (GDPs, identified as highly reactive carbonyl compounds), low pH, and hyperosmolality (1,6,7). In addition, uremic toxins and another reactive substances can change the physicochemical properties of peritoneum.

Uremic stage can modify the efficiency of renal replacement therapy, especially changes in the structural and transport properties of peritoneum. Concentration of one GDP, methylglyoxal, has been found to be elevated in the blood of uremic patients with chronic renal failure, associated with a decline in levels of antioxidant factors in serum. This GDP

is a metabolic hazard and a potent glycating agent in the body, and an important precursor of advanced glycation and lipoxidation end-products (AGEs and ALEs). It has been speculated that, *in vivo,* GDPs are a larger source of AGEs than is glucose itself (8). Moreover, positive correlations have been noted between mitochondrial DNA copies in the peripheral leukocytes of continuous ambulatory PD patients and both elevated oxidative stress and higher peritoneal urea clearance (9).

The polycationic peptide protamine sulfate (about 5000 Da in molecular weight) contains about 67% arginine. This drug is administered to reverse the large dose of heparin given during surgeries. It can bind to heparin to create a stable ion pair that has no anticoagulant activity (10). The influence of protamine on the properties of the peritoneal membrane has been little studied. It is well documented that some arginine-rich peptides improve epithelial absorption of insulin from the intestine to the systemic circulation, without toxic effects (11). A negatively charged peritoneal surface can be neutralized by cationic protamine, causing modification of membrane permeability (12,13).

Glucose is not a stable compound in dialysis solution (75 – 214 mmol/L, 15 – 40 times physiologic concentrations). It can undergo a variety of spontaneous degradation reactions, intensified during heat sterilization of PD solution, resulting in the formation of new reaction products (GDPs). Most of these compounds are toxic low-molecular-weight aldehydes such as glyoxal, methylglyoxal, 2-furaldehyde, and formaldehyde (7,14,15). Recently, studies to improve the biocompatibility of PD fluid (for example, to lower the concentration of GDPs) have been intensifying (3,16). The cytotoxicity exerted by some GDPs on the peritoneal membrane has been well documented, but the mechanism is not well understood (3,7,14,15). The present study therefore examined whether a change in charge (with use of protamine sulfate) and short-term exposure of the peritoneum to aldehydes (glyoxal, methylglyoxal) influence the transport coefficients for a neutral, low-molecular-weight toxin such as urea.

Material and methods

The experiments were carried out using fragments of parietal peritoneum from New Zealand male rabbits placed into a modified Ussing chamber system (Local Ethics Committee for Animal Research, Poznań, Poland, approval 22/2006). The active membrane surface area was 1.1 cm^2. The tissue was connected by peris-taltic pump to a fluid reservoir containing Hanks solution (136.88 mmol/L NaCl, 5.36 mmol/L KCl, 4.16 mmol/L $NaHCO_3$, 1.26 mmol/L $CaCl_2$, 0.44 mmol/L KH_2PO_4, 0.34 mmol/L $Na_2HPO_4 \times 12$ H_2O, 0.41 mmol/L $MgCl_2 \times 7$ H_2O) circulated at the rate of 11 mL/min. Adequate oxygen content in the medium and a constant pH of 7.4 were both maintained by continuous bubbling of a gas mixture consisting of CO_2 50 mL/L and O_2 950 mL/L. The entire system was maintained at 37°C in a thermostat box during the experiments (4).

The transfer rate of urea [initial concentration gradient: 20 mg/dL (POCH, Gliwice, Poland)] from the interstitial to the mesothelial side (I→M) of the peritoneal membrane and in the opposite direction (M→I) was studied in four separate series:

- Control conditions (120 minutes)
- Before (15 – 60 minutes) and after (75 – 120 minutes) addition of protamine sulfate 0.005 g/dL on the mesothelial side of the peritoneum
- Before (15 – 60 minutes) and after (75 – 120 minutes) addition of glyoxal trimer dihydrate 0.01 g/dL on the mesothelial side of the peritoneum
- Before (15 – 60 minutes) and after (75 – 120 minutes) addition of methylglyoxal 0.001 g/dL on the mesothelial side of the peritoneum

Sampling of the medium was carried out at regular 15-minute intervals. The urea concentration was determined using the enzymatic test with urease and glutamate dehydrogenase (Cormay, Lublin, Poland).

A mathematical model of mass transport was used to estimate a diffusive permeability coefficient *P* (scaled to the surface area of the membrane investigated *in vitro*) for the examined specimen. Changes in *P* attributable to experimental modifications were determined as percentages of the control value for each individual experiment (that is, separately for each sample of peritoneal membrane) and are presented as mean ± standard error of the mean (SEM) for the entire series. In this way, the initial part of an experiment with a particular membrane sample served as a control for the second part. Statistical analysis was performed using Shapiro–Wilks and Wilcoxon matched-pairs rank-sum tests and the Student *t*-test for paired data

(Statistica 7.1: StatSoft, Tulsa, OK, U.S.A.). A p value less than 0.05 was considered significant.

Results

In control conditions, during the 120 minutes of the study without protamine sulfate, glyoxal, or methylglyoxal, the rates of urea transport directed from the interstitial to the mesothelial side of membrane and in the opposite direction remained constant. For the cases of I→M and M→I, the values of $P \pm$ SEM were 2.293 ± 0.211 cm/s and 2.621 ± 0.457 respectively (×0.0001). The introduction of protamine sulfate (0.005 g/dL) and methylglyoxal (0.001 g/dL) at 60 minutes of the experiment did not alter bidirectional urea transfer. However, glyoxal (0.01 g/dL) resulted in a decrease of about 12% ($p < 0.01$) in urea M→I transport, with a nonsignificant reduction in the opposite direction (Figure 1).

Discussion

Few studies have been conducted regarding the mechanism of urea transfer through the peritoneal membrane and the factors that can change the transfer process. Clinical and experimental analyses show that this toxin is rapidly transported from the blood to peritoneal cavity mainly across small pores (1,2). Transfer through the peritoneum occurs bidirectionally, depending on the concentration gradient.

In clinical studies, small uremic toxins are transported much more rapidly than are protein or glucose polymer molecules. In the present work, we analyzed the passage of urea, a small, neutral solute (molecular radius: 2.6 Å; molecular weight: 60 Da) with osmotic properties. Peritoneal transport of urea in our *in vitro* study was stable for 120 minutes of the experiment. When we compare the transfer parameters for other small toxins—uric acid or creatinine—examined in the same experimental system, results are similar (4,16,17). Values of P for urea [mean: 2.457 ± 0.332 cm/s (×0.0001)] and the other two small solutes are about 10 times those for macromolecules such as albumin or icodextrin (4,17).

During a 2-hour PD dwell in rabbits, 5 mg/dL protamine did not change the transfer of small, neutral molecules such as urea after 60 minutes of incubation; however, it did enhance transperitoneal passage of albumin (a negatively charged compound). Availability of anionic sites is probably the crucial limiting factor, because neutralization of protamine with heparin prevents the changes in transperitoneal transfer of the negatively charged protein (13). In other studies, protamine concentrations between 0.5 mg/dL and 3 mg/dL did not alter permeability to urea, but lowered the transfer of inulin. At concentrations from 3 mg/dL to 7.5 mg/dL, protamine increased permeability for small and middle solutes by 50% and 20% respectively. Mesothelial cells revealed a partially reversible loss of microvilli and a minor degree of disorganization of submembranous cytoplasmic microfilaments, without changes in the intramembranous structure of occluding junctions. At a concentration of 10 mg/dL protamine, permeability for inulin doubled without a comparable effect on permeability for urea, associated with an irreversible disruption of occluding junctions (12). In the *in vitro* study with rabbit parietal peritoneum, 5 mg/dL protamine sulfate did not affect urea transfer, but did increase transport of the large-molecule glucose polymer icodextrin (18). The effect of the examined polycationic polypeptide on

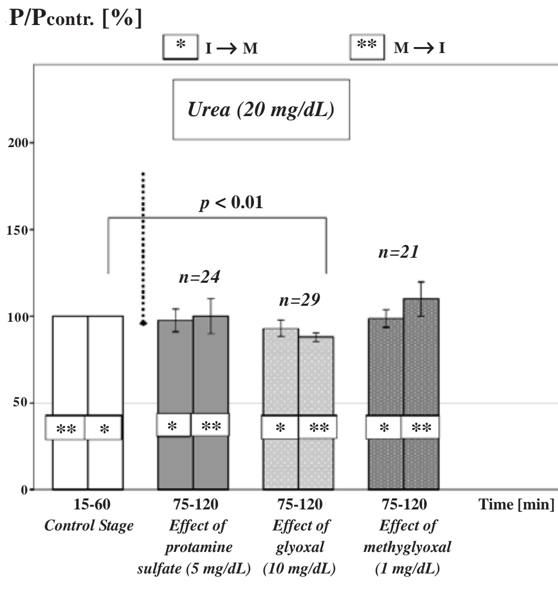

FIGURE 1 Changes of diffusive permeability (P) for urea in rabbit peritoneum, expressed as a percentage of the control (contr.) value ± standard error of the mean obtained before the application of protamine sulfate, glyoxal, or methylglyoxal on the mesothelial side of the membrane at 60 minutes of the experiment. I = interstitial side of the peritoneal membrane; M = mesothelial side of the peritoneal membrane; n = number of experiments.

peritoneal transport of small solutes is highly dependent on its concentration.

In the literature, the effects of GDPs on transperitoneal transport are variable. Studies have concentrated on the influence of selected GDPs on morphologic and secretory changes of peritoneum (3,6,7,19). Peritoneal dialysis solutions with high levels of GDPs promote DNA damage in human peritoneal mesothelial cells (they decrease the mesothelial cell mass indicator cancer antigen 125) and increase cell death (3,20). Moreover, reactive carbonyl compounds were found to be able to suppress proliferation of cultured human mesothelial cells (7). The absence of mesothelium lining in PD patients has been related to cumulative exposure of the peritoneum to dialysis fluid with GDPs (21).

In cultured rat mesothelial cells exposed to fluid containing methylglyoxal, peritoneal fibrous thickening with proliferation of mesenchymal-like mesothelial cells has been observed. In the present study, the expression of transforming growth factor β, collagen 1, vascular endothelial growth factor (VEGF), and AGE receptor also increased. In the case of formaldehyde-treated rats, the peritoneum was thickened, with sparse collagen fibers, but mesenchymal-like mesothelial cells were not observed (19). Glucose degradation products also play an important role in inducing VEGF (a potent angiogenic compound) production by cultured human mesothelial cells. This factor is well documented to play an important role in the change of peritoneal permeability (6,20). The foregoing data have proved that GDPs indirectly influence transperitoneal transfer.

After a 4-hour PD procedure in rats using a mixture of GDPs with glyoxal and methylglyoxal (in doses similar to those in the present *in vitro* studies), albumin clearance from the peritoneal cavity was reduced. Moreover, a concomitant thinning of the submesothelial tissue, but no difference in cell density in the tissue, was observed (14). Reactive carbonyl aldehyde caused depolarization and decline in the permeability of mitochondrial membrane of rat liver. In rat pancreatic cells, GDPs increased cytosol calcium concentration (7,15). In the present *in vitro* study, introduction of glyoxal (10 mg/dL) into the circulating medium with urea caused a lowering of uremic toxin transfer from the mesothelial to the interstitial side of the peritoneum by 12% over 60 minutes, with a nonsignificant reduction in the opposite direction. Under the same conditions, the addition of methylglyoxal (1 mg/dL) did not change transport parameters. However, in our previous studies, both of these aldehydes diminished the transport of creatinine (22,23). Differences in the effect of methylglyoxal on urea and creatinine transport are probably connected to the individual physicochemical characteristics of these uremic toxins—specifically, the osmotic properties of urea and the specific molecular shape of creatinine.

Conclusions

The results obtained here show that protamine sulfate and methylglyoxal do not affect, but that glyoxal modifies, the diffusive permeability of peritoneum *in vitro.* The causes of these effects are currently unknown, but may be observed *in vivo* during PD and can change the efficiency of renal replacement therapy.

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