Advances in Peritoneal Dialysis, Vol. 20, 2004

Intraperitoneal Enalapril Ameliorates Morphologic Changes Induced by Hypertonic Peritoneal Dialysis Solutions in Rat Peritoneum

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Peritoneal fibrosis (PF) is one of the most serious causes of technique failure in long-term peritoneal dialysis (PD). Although the mechanisms responsible for the genesis of PF are not well understood, angiotensin II is known to promote fibrosis and inflammation in various tissues and angiotensin converting enzyme inhibitors (ACEIs) have been shown to attenuate those effects.

We previously showed that ACEIs have beneficial effects on peritoneal alterations induced by hypertonic (3.86% glucose) PD solutions. In the present study, we investigated the local effects of intraperitoneal (IP) enalapril on peritoneal alterations induced by 3.86% glucose PD solution in rats on chronic PD.

*One week after peritoneal catheter insertion, 23 non uremic male rats were randomly divided into two groups: group A (*n = *11) received 20 mL 3.86% PD solution twice daily, and group B (*n = *12) received 20 mL 3.86% PD solution containing 1 mg/L enalapril twice daily. After 4 weeks of such infusions, we measured net ultrafiltration (UF) volume and obtained samples of visceral peritoneum from the liver for thickness measurement. Net UF was significantly higher (6.6 ± 0.2 mL vs. 5.6 ± 0.2 mL) and peritoneal thickness was significantly lower (30 ± 5 μm vs. 52 ± 0.8 μm) in group B.*

We conclude that intraperitoneal enalapril (an ACEI) protects the peritoneal membrane from the effects of hypertonic glucose. This protection might be mediated by enalapril's interference with angiotensin though inhibition of cytokine overexpression.

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Key words
Enalapril, hypertonic glucose, inflammation, ultrafiltration failure

Introduction

During continuous ambulatory peritoneal dialysis, various morphologic changes take place in the peritoneum, including mesothelial denudation, interstitial fibrosis, neovascularization, and vascular alterations such as replication of basement membrane, fibrosis, and hyalinization of the vascular wall (1–4). Among the suggested causes for these histologic and functional alterations are recurrent peritonitis, the influence of plasticizers and advanced glycosylation end-products, and response to the unphysiologic nature of peritoneal dialysis (PD) solutions, in particular the high glucose content, hypertonicity, lactate, and low pH (2–4).

Although the mechanisms underlying the alterations are not completely understood, growth factors and cytokines secreted by mesothelial cells and macrophages have been shown to be associated with peritoneal fibrosis (5). Dobbie (6) suggested that chronic irritation from PD solutions and severe or prolonged peritonitis are the initiating events that lead to peritoneal fibrosis and injury to mesothelial cells. Damaged mesothelial cells can cause peritoneal fibrosis by secreting extracellular matrix macromolecules consisting of collagen, fibronectin, laminin, proteoglycans, and cytokines, including transforming growth factor beta 1 (TGFβ1) and interleukin-1 (IL-1).

Angiotensin II, the main peptide of the renin–angiotensin system, is an oligopeptide consisting of 8 amino acids. It is formed from its precursor, angiotensinogen, by a series of two enzymatic

cleavages. It is now clear, however, that extrarenal renin–angiotensin systems exist and that angiotensin II can be synthesized at a variety of sites, including the kidney, vascular endothelium, adrenal glands, and brain. Local angiotensin II production is presumed to be important for the regulation of local processes that are activated by local factors such prostaglandin, nitric oxide, and endothelin.

Angiotensin II is considered to be a growth factor that regulates cell proliferation, apoptosis, and fibrosis. In models of renal injury, the use of angiotensin converting enzyme inhibitors (ACEIs) or angiotensin receptor (AR) blockers to inhibit the action of angiotensin II leads to reductions in proteinuria, infiltration by inflammatory cells, fibrosis, and gene expression of matrix proteins and growth factors (7–9). Renin–angiotensin system blockers are commonly used in the treatment of hypertension and proteinuria in humans (10).

Angiotensin II stimulates macrophages and fibroblast-like cells to secrete TGFβ1. For instance, a perivascular or interstitial fibrosis (or both) accompanies chronic elevation of either circulating angiotensin II or aldosterone (11) and, in the case of angiotensin II, occurs in response to abnormal vascular permeability and escape of macromolecules (12). Angiotensin II also regulates the synthesis of proinflammatory cytokines [tumor necrosis factor alpha (TNFα) and IL-6] and chemokines [monocyte chemoattractant protein-1 (MCP-1)] in the kidney (13).

Macrophages in the peritoneal cavity seem to participate in the process of peritoneal fibrosis through the production of various cytokines and growth factors. The mechanisms by which monocytes enter the peritoneal cavity are not fully understood. The potent chemokine MCP-1, which has a considerable specificity for monocytes, has been reported to play a key role in the recruitment of monocytes toward the peritoneal cavity (14). In addition to chemotactic activity, MCP-1 can induce calcium flux and respiratory burst activity and can also regulate adhesion molecule expression and cytokine production in monocytes. It is also known that MCP-1 can stimulate TGFβ1 production in lung fibroblasts (15,16).

We previously showed that ACEIs have beneficial effects on peritoneal alterations induced by hypertonic PD solutions (17–19). In the present study, we investigated the local effects of enalapril given intraperitoneally on peritoneal alterations induced by hypertonic PD solutions in rats on chronic PD.

Materials and methods

We performed the study in non uremic male albino Wistar rats weighing 280 – 330 g. The rats were housed in polycarbonate cages and were fed a standard laboratory diet. They were also given free access to water. The temperature was maintained at 30°C with a 12-hour light/dark cycle. Peritoneal catheters were implanted into 23 rats under ether anesthesia.

For the first week, all rats were injected intraperitoneally with Dianeal 1.36% glucose (Baxter Healthcare SA, Castlebar, Ireland) supplemented with antibiotics [gentamicin 10 mg/L (Polfa, Tarchomin, Poland) and cefazolin 100 mg/L (Eli Lilly, Florence, Italy)]. The fluid was allowed to absorb.

One week after peritoneal catheter insertion, the rats were randomly divided into two groups: group A (n = 11) received 20 mL of hypertonic (3.86%) PD solution twice daily, and group B (n = 12) received 20 mL hypertonic PD solution containing 1 mg/L enalapril (Enap: Krka, Novo Mesto, Slovenia) twice daily. Both solutions were again also supplemented with antibiotics.

Earlier, we had performed an acute pilot study to determine intraperitoneal enalapril dosage. We compared doses of 0.1 – 25 mg/L. Based on the results of that study, we chose the 1 mg/L concentration used in the present study.

Dialysate cell count was measured weekly and dialysate samples were taken at the 4th hour of a dwell for measuring selected inflammatory parameters at the beginning, middle, and end of the study. Dialysate nitrites (an index of NO synthesis) was measured according to the method presented by Gilliam *et al.* (20). We measured MCP-1 using an ELISA kit (Biosource, Camarillo, CA, U.S.A.).

After 4 weeks, we performed a 1-hour peritoneal equilibration test (PET) in each rat, using 20 mL of 3.86% PD solution. Then, under ether anesthesia, the rats were humanely killed by bleeding. The peritoneal cavity was opened to estimate the presence and severity of intraperitoneal adhesions and morphologic changes. Samples of liver and parietal peritoneum were also collected for morphology analysis.

Peritoneal transport was determined using dialysate-to-plasma (D/P) concentration ratios of urea nitrogen and total protein. In the samples, the urea

nitrogen concentrations were determined by an enzymatic method (kit numbers A-371 and A-291 from Analco, Warsaw, Poland). Total protein concentration was measured by the Lowry colorimetric method.

Evaluation of peritoneal adhesions

Intensity of the peritoneal adhesions was evaluated according to 3-point scale:

- 1 point: thin adhesion (1 – 2 mm) between the catheter and the peritesticular fat pad
- 2 points: medium adhesion (3 – 5 mm) between catheter and the peritesticular fat pad
- 3 points: thick adhesion (>5 mm) between the catheter and the peritesticular fat pad or adhesions between intraperitoneal organs

In cases in which the peritoneal cavity of a rat showed more than one adhesion, we totaled the point score for the various adhesions.

Pathology examination

Samples from the liver and abdominal wall were obtained and fixed with 10% formaldehyde solution in phosphate-buffered saline. Paraffin-embedded tissue sections were stained using the van Gieson method for visualization of collagen.

As in our previous studies (17–19), we used a light microscope supported by digital image analysis equipment (Lucia 4.6: Laboratory Imaging, Prague, Czech Republic) for the thickness measurements. Results are expressed as the mean of 10 measurements.

Statistical analysis

Results are reported as mean ± standard error of the mean. The statistical analyses were performed using analysis of variance, unpaired *t*-test, and the Mann–Whitney test. A *p* value of less than 0.05 was considered significant.

Results

Tables I – III summarize our findings. We found significant functional and morphologic differences between group A and group B.

After 4 weeks' exposure to hypertonic PD solutions, peritoneal functions decreased in group A (higher D/P urea and protein). Peritoneal functions were preserved in group B ($p < 0.05$), which showed a lower dialysate cell count (1163 ± 109 vs. 1552 ± 309, $p > 0.05$) and lower MCP-1 levels (289 ± 25 pg/mL vs. 226 ± 12 pg/mL, $p < 0.05$) than those found in group A. A significant correlation (52%) was observed between the dialysate cell count and the MCP-1 level ($p < 0.05$). No difference in dialysate NO levels was noted between the two groups (Table II).

TABLE I Functional and macroscopic findings

	Without enalapril (n=11)	*With enalapril (n=12)*
Net UF volume (mL)	5.68±0.17	6.57±0.16
WBC (cells/mm^3)	1552±309	1163±109
D/P urea	0.76±0.03	0.61±0.02
D/P protein (×1000)	61±2.1	54±1.3
Rats with liver changes (*n*)	11/11 (100%)	7/12 (58%) [a]
Rats with adhesions (*n*)	9/11 (82%)	5/12 (42%) [a]
Adhesions (*n*)	13	7
Rats with severe (score ≥ 2) adhesions (*n*)	8 (61%)	2 (28%)

[a] $p < 0.05$, without enalapril versus with enalapril.
UF = ultrafiltration; WBC = white blood cells; D/P = dialysate-to-plasma concentration ratio.

TABLE II Dialysate levels of white blood cells (WBC), monocyte chemoattractant protein 1 (MCP-1), and NO at various points during the study period

	Start	*Middle*	*End*
Without enalapril			
WBC (cells/mm^3)	1528±141	1595±107	1591±147
MCP-1 (pg/mL)	590±44 [a]	425±34 [a]	289±25 [a]
NO (µmol/L)	7±0.4	6.73±0.3	6.7±0.1
With enalapril			
WBC (cells/mm^3)	1633±192	1327±137	1413±140
MCP-1 (pg/mL)	403±25	325±36	226±12
NO (µmol/L)	7.25±0.4	6.77±0.3	6.5±0.2

[a] $p < 0.05$, without enalapril versus with enalapril.

The adhesion point score (13 vs. 7) and the prevalence of intraperitoneal adhesions (82% vs. 42%) were higher in group A than in group B ($p < 0.05$). Group A also showed a high incidence of severe adhesions as compared with group B (61% vs. 28%, $p > 0.05$). Overall, the severity of the adhesions was less in group B than in group A. In group B, only 2 rats had adhesions evaluated above 2 points (see Table I and Figure 1).

Peritoneal thickness was significantly lower in group B than in group A (30 ± 5 µm vs. 52 ± 8 µm, Figure 2). In the histology examination, less

TABLE III Histology examination results in the two groups

	Visceral peritoneum (liver)			Parietal peritoneum		
	Thickness	*INF*	*VAS*	*Thickness*	*INF*	*VAS*
Without enalapril	52±8 [a]	1	0.72	219±20 [a]	1.81	2.1
With enalapril	30±5	0.91	0.58	143±11	1.66	2

[a] $p < 0.05$, without enalapril versus with enalapril.
INF = inflammation; VAS = vascularization.

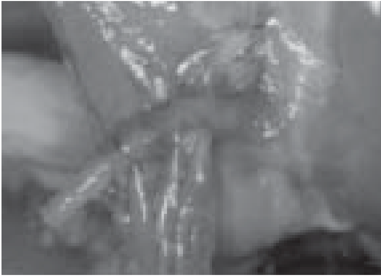

FIGURE 1 Severe adhesions induced by high glucose.

inflammation and vascularization was seen in group B ($p > 0.05$, Table I).

Discussion

Pharmacologic interventions in peritoneal solute transport may be used to evaluate functional properties and to prevent peritoneal fibrosis, augmenting the viability of the peritoneal membrane. Previously, using animal models, we investigated the effect of ACEIs or AR blockers (or a combination of both) on peritoneal alterations induced by hypertonic PD solutions. In a rat model of once-daily injection of 3.86% PD fluid for 4 weeks, we found that

- oral administration of enalapril ameliorates changes in peritoneal function and morphology (17),
- oral administration of lisinopril (an ACEI) and valsartan (an AR blocker) have similar beneficial effects on peritoneal function and morphology (18), and
- oral administration of quinapril plus valsartan has no synergistic effect on peritoneal alterations induced by 3.86% PD solutions (19).

With regard to histology, we found less cell infiltration and less vascularization in all treated groups.

Based on those findings, we concluded that, by inhibiting the overexpression of cytokines (that is, TGFβ1 and VEGF), renin–angiotensin system blockade ameliorates peritoneal injury induced by hyper-

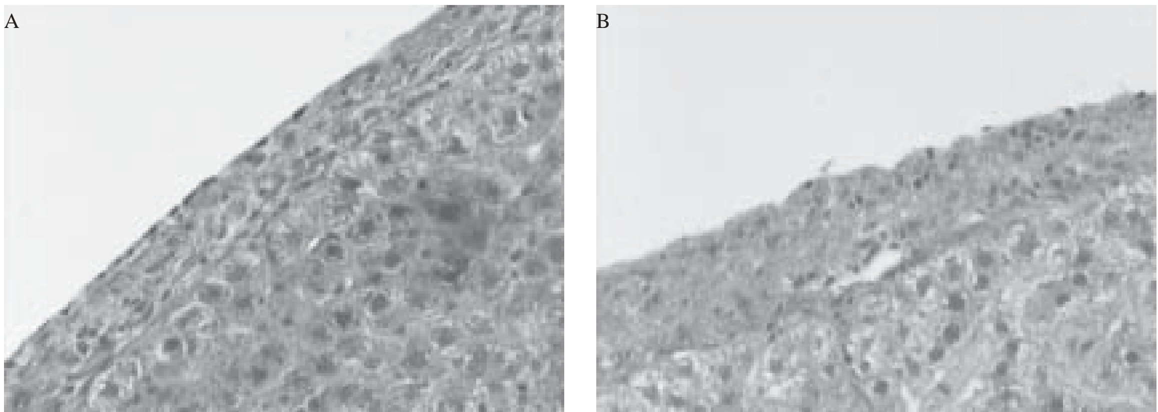

FIGURE 2 Van Gieson stain of visceral peritoneum (liver). (A) Group with enalapril. (B) Group without enalapril.

tonic PD solution and that ACEIs or AR blockers, or both, might preserve the viability of peritoneum in continuous ambulatory PD patients over long periods.

The present study shows that hypertonic PD solutions play a major role in inducing peritoneal membrane failure and peritoneal fibrosis. Rats exposed to hypertonic PD solutions showed structural deterioration of the peritoneal membrane, including increased membrane thickness, neovascularization, inflammatory cell infiltration, and fibrosis. Those alterations induced by hypertonic PD solutions were partially prevented by intraperitoneal enalapril treatment, which reduced damage to the peritoneal structure. Intraperitoneal adhesions, vascularity, fibrosis, and inflammatory cell infiltration were less in group B than in group A. Those results and the results of Sawada *et al.* (21) indicate that local ACE inhibition is important for preserving peritoneal membrane morphology and preventing fibrosis.

Given the role of angiotensin II in promoting TGFβ1 and MCP-1 expression (and the fibrogenic potential of those cytokines), the use of pharmacologic strategies (that is, ACEIs) to interfere with angiotensin II formation or with its angiotensin I–receptor binding needs to be addressed.

Activated macrophages isolated from peritoneal exudates formed after instillation of mineral oil into the peritoneal space have been shown to express angiotensin II receptors (22). Fibrotic disease is characterized by an accumulation of extracellular matrix (ECM). Overexpression of TGFβ1 has been shown to be responsible for such accumulation through its actions in inducing production of ECM, inhibiting degradation of ECM, and increasing integrin expression, resulting in matrix deposition (5).

The mechanisms by which hypertonic PD solutions induce these structural and functional alterations in the peritoneum are not completely understood. Exposure to high glucose increases dialysate MCP-1 levels, which decrease with exposure time. That finding confirms earlier reports. However, Lee *et al.* showed that high glucose induces MCP-1 expression partly via the tyrosine kinase–AP-1 pathway in peritoneal mesothelial cells (16). Cytokines and chemokines—including MCP-1—are secreted in response to major abdominal operations (23) and are related to intra-abdominal adhesion formation.

In the present study, we showed that high glucose concentrations increased MCP-1 expression and intraperitoneal adhesions. Enalapril given intraperitoneally prevented the lesions, probably by reducing overexpression of MCP-1. We also found a lower dialysate cell count in group B, which may also be related to lower MCP-1 levels. That hypothesis is supported by the significant correlation between MCP-1 level and dialysate white blood cell count that we observed.

Conclusion

Our results show that commercially available hypertonic PD solutions cause morphologic and functional alterations in rat peritoneum under the experimental circumstances that we used. Enalapril (an ACEI), given intraperitoneally, protects the peritoneal membrane from the effects of hypertonic glucose. The protective effect may be mediated by a reduction in inflammation and in angiotensin II–induced overexpression of MCP-1.

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Aquaporin-1 Is Recruited to the Plasma Membrane by Hyperosmotic Stimuli via a Protein Kinase A–Dependent Pathway in Rat Peritoneal Mesothelial Cells

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Aquaporin-1 (AQP1) has been reported to play an important role in water permeability in peritoneal dialysis. To determine the mechanism involved in this process, we used cultured rat peritoneal mesothelial cells (RPMCs) to examine the glucose-induced translocation of AQP1 to the plasma membrane.

Cultured RPMCs obtained from male Sprague–Dawley rats were incubated in a combination of Dulbecco modified Eagle medium (DMEM) and F12 medium at 37°C for 15 minutes. The plasma membrane of the RPMCs was separated by Percoll gradient, and the quantity of AQP1 in the membrane fraction was determined by Western blot analysis. The amount of AQP1 was significantly increased by the addition of 5% glucose (139.5% ± 38.7% of control, $p < 0.05$*) or of dibutyryl cyclic adenosine monophosphate (db-cAMP), a cAMP analog to the medium (139.5% ±21.9% of control,* $p < 0.05$*). However, glucose-induced enhancement of AQP1 disappeared with the addition of H-89, a protein kinase A (PKA)–specific inhibitor (103% ± 17.5% of control,* $p < 0.05$ *as compared with 5% glucose).*

We also examined the effect of 5% glucose on PKA activity separately in the cytosol fraction, the crude membrane fraction, and the pure plasma membrane fraction. In the cytosol fraction of 5% glucose–stimulated RPMCs, PKA activity was decreased (70.5% ± 11.5% of control, $p < 0.01$*), but in the crude membrane fraction, it was significantly increased (143.9% ± 52.9% of control,* $p < 0.01$*). In the pure plasma membrane fraction, PKA activity did not change. From those findings, we hypothesize that 5% glucose augments the PKA-dependent translocation of AQP1 to the plasma membrane, mediated by PKA translocation to the intracellular AQP1 store.*

Key words
Aquaporin-1, translocation, peritoneal mesothelial cells, protein kinase A

Introduction

Peritoneal dialysis (PD) is a therapy that focuses on quality of life in end-stage renal disease (ESRD) patients, and PD is applicable for patients of all ages. However, many PD patients eventually change to hemodialysis (HD) therapy. The main cause of such switching is poor ultrafiltration attributable to peritoneal damage from long-term PD. Maintenance of good ultrafiltration is therefore one of the most important factors in extending the period of PD (1).

Rippe *et al.* (2) used computer simulation analysis to proposed a "three-pore model" of fluid transport in the peritoneum. The model postulates that size-selective "ultrasmall pores" pass only water. In 1992, Preston *et al.* (3) cloned a water channel from red blood cells and named this membrane protein "aquaporin-CHIP" [aquaporin-1 (AQP1)]. Lately, it has become clear that mRNA for AQP1, AQP3, and AQP4 are all expressed in human peritoneal tissues, and expression of AQP1 has been reported to appear stronger than that of AQP3 and AQP4 under the same polymerase chain reaction (PCR) conditions (4).

Recently, AQP1 has been suggested to be equivalent to the ultrasmall pores. The importance of AQP1 for ultrafiltration has also been reviewed in several studies. Yang *et al.* (5) reported that the osmotic water permeability of the peritoneal barrier was reduced in

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AQP1-gene knockout mice. Carlsson *et al.* (6) reported that inhibition of AQP1 by mercury reduced ultrafiltration in a rat model of PD. Furthermore, Akiba *et al.* (7) reported that mRNA of AQP1 was increased by hyperosmotic stimuli such as glucose and mannitol during 24 hours' exposure in a primary culture of rat peritoneal mesothelial cells (RPMCs), and Lai *et al.* (8) reported that mRNA of AQP1 was increased in human peritoneal mesothelial cells after 3 hours' exposure to hyperosmotic stimuli (such as glucose). However, these studies were long-term exposure experiments, in which the increase in AQP1 was suggested to be a result of enhanced protein synthesis accompanied by an increase in gene expression.

Peritoneal dialysate contains glucose as an osmolyte to create an osmotic gradient for ultrafiltration. It is widely recognized that, just after infusion of peritoneal dialysate into the peritoneal cavity, the ultrafiltration volume varies according to the osmotic gradient generated by the glucose (9). That finding can be explained only by AQP1 translocation to the plasma membrane rather than by AQP1 protein synthesis as we previously reported (10). In the present study, we therefore used cultured RPMCs to try to elucidate the mechanism of hyperosmosis-induced AQP1 translocation to the plasma membrane.

Materials and methods

Primary culture of RPMCs

We obtained RPMCs from the parietal mesothelium of 250 – 300 g male Sprague–Dawley rats. The resected parietal peritoneum was incubated for 30 minutes at 37°C in a combination of Dulbecco modified Eagle medium and F12 medium (DMEM/F12: Gibco BRL, Rockville, MD, U.S.A.) containing type-I collagenase (Gibco). The rat peritoneal mesothelial cells were cultured primarily using 10% fetal calf serum containing DMEM/F12 as described by Hjelle *et al.* (11).

Preparation of plasma membrane

For the experiment, 3 – 7 generations of RPMCs were applied to avoid transformation of the cells. All experiments were performed using the RPMCs in detached (floating) condition. The RPMCs were incubated at 37°C for 15 minute with DMEM/F12 (control), with osmolality-adjusted culture medium (5% glucose), with dibutyryl cyclic adenosine monophosphate (db-cAMP, 1 mmol/L) as a protein kinase A (PKA) activator, and with 5% glucose with H-89 (30 μmol/L) as a PKA inhibitor. After incubation, the RPMCs were gently centrifuged to remove the culture medium. A motor pestle homogenizer was then used to homogenize the gathered RPMCs in homogenization buffer [HEPES 12 mmol/L and mannitol 300 mmol/L, titrated to pH 7.6 with 1 mol/L tri(hydroxymethyl)aminomethane (Tris)]. The homogenate was centrifuged at 2500*g* for 15 minutes at 4°C, and the supernatants were centrifuged again at 48,000*g* for 30 minutes at 4°C. The upper part of the pellet was re-suspended in the same homogenization buffer. Percoll solution was then added to create the gradient, and the mixture was centrifuged again in a swing rotor at 48,000*g* for 30 minutes at 4°C. The proteins were electrophoresed using a 12% sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) process, transferred to polyvinylidene fluoride membranes (Immobilon: Millipore Corporation, Bedford, MA, U.S.A.), and incubated with mouse monoclonal anti-AQP1 antibody (Chemicon International, Temecula, CA, U.S.A.). An immunoreactive band was visualized by chemiluminescence using anti-mouse horseradish peroxidase and the Western blot detection system (ECL Plus substrate: Amersham Pharmacia Biotech, Little Chalfont, U.K.) on X-ray film. The bands of AQP1 were quantitated by densitometry.

Measurement of PKA activity

We examined the effect of 5% glucose on PKA activity separately in three cell fractions: cytosol, crude membrane, and pure plasma membrane. The RPMCs were incubated with DMEM/F12 (control) or with osmolality-adjusted culture medium (5% glucose) for 1 minute at 37°C in floating condition. Cells were centrifuged and homogenized in ice-cold sample preparation buffer [50 mmol/L Tris–HCl (pH 7.5), 5 mmol/L ethylenediaminetetraacetic acid (EDTA), 10 mmol/L ethyleneglycoltetraacetic acid (EGTA), 50 mmol/L 2-mercaptoethanol, 1 mmol/L phenylmethylsulfonyl fluoride, 10 mmol/L benzamidine (10 μg/mL)]. The homogenates were centrifuged at 2500*g* for 15 minutes at 4°C, and the supernatants were centrifuged again at 100,000*g* for 1 hour at 4°C. The resulting supernatant was obtained as cytosol fraction. The pellet obtained as crude membrane fraction was rehomogenized with sample preparation buffer.

The PKA activity was measured using a non radioactive PKA assay system (Medical and Biological Laboratories, Nagoya, Japan) according to the manufacture's instructions.

Statistical analysis

The results are expressed as mean ± standard deviation. Statistical analysis was carried out using the StatView computer program (SAS Institute, Cary, NC, U.S.A.), version 5.0, on a PC. A p value of less than 0.05 was considered significant. The Student t-test was used for the PKA activity of the three cell fractions, and analysis of variance was used for the effects of PKA activation and inhibition on the amount of AQP1 protein in plasma membrane.

Results

Identification of RPMCs

The RPMCs showed the cobblestone-like appearance that is the typical morphologic characteristic in subconfluent condition. Furthermore, immunostaining revealed the cells as positive for cytokeratin 18 and vimentin, and negative for von Willebrand factor. Those findings validated the cells as mesothelial cells.

Effects of PKA activation on amount of AQP1 protein in plasma membrane

The RPMCs were incubated for 15 minutes at room temperature with normal culture medium (control group), with osmolality-adjusted culture medium (5% glucose group), and with 1 mmol/L db-cAMP, a PKA activator, added to the culture medium (PKA activator group). In the plasma membrane prepared from the RPMCs, AQP1 was recognized as a band at 28 kDa. [Figure 1(A) shows a representative Western blot.] The amount of AQP1 protein was significantly enhanced in the PKA activator group (139% ± 38.7% of control, $p < 0.05$) and in the 5% glucose group (139% ± 21.9% of control, $p < 0.05$) as compared with the control group.

Influence of a specific PKA inhibitor, H-89, on the quantity of AQP1 protein in the plasma membrane fraction of RPMCs

To clarify whether increased abundance of AQP1 on the plasma membrane was induced by stimulation of the PKA pathway or by a specific effect of db-cAMP,

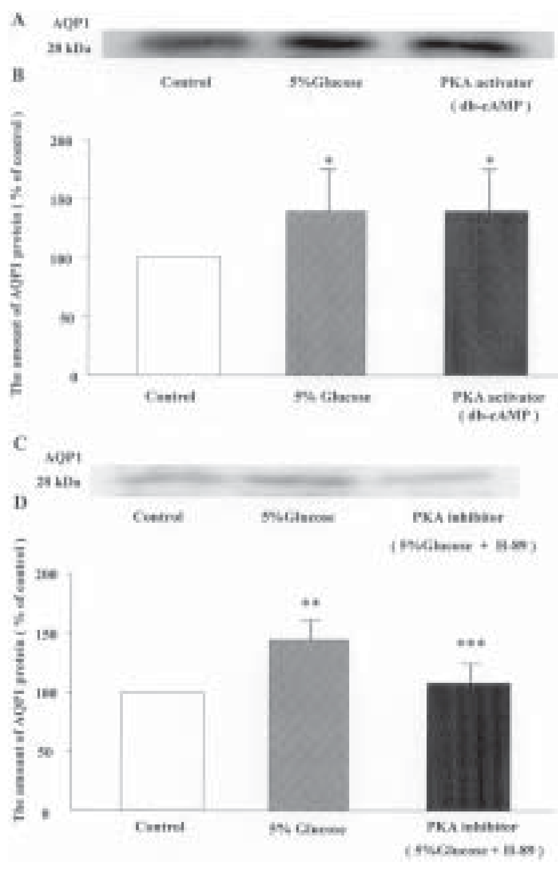

FIGURE 1 Effect of 5% glucose and a protein kinase A agonist or inhibitor on the quantity of aquaporin-1 (AQP1) protein in the plasma membrane fraction of rat peritoneal mesothelial cells. (A) Representative Western blot determination of AQP1 in the plasma membrane fraction. (B) Densitometric analysis of blots incubated with 5% glucose or dibutyryl cyclic adenosine monophosphate (db-cAMP). * $p < 0.05$ as compared with control [Scheffe test after analysis of variance (ANOVA); $n = 6$ for each group]. (C) Representative Western blot determination of AQP1 in the plasma membrane fraction. (D) Densitometric analysis of blots incubated with or without H-89. ** $p < 0.05$ as compared with control. *** $p < 0.05$ as compared with 5% glucose (Scheffe test after ANOVA; $n = 6$ for each group).

we performed the experiment under the same protocol, but with H-89 instead of db-cAMP. The amount of AQP1 protein in the 5% glucose group was significantly higher than in the control group (142% ± 32.7% of control, $p < 0.05$); however, the increase in the amount of AQP1 protein was completely abolished by H-89 (103% ± 17.5% of control, $p < 0.05$ as compared with 5% glucose group; Figure 1).

Change in PKA activity in cytosol fraction, crude membrane fraction, and pure membrane fraction of RPMCs

Figure 2(A) shows the effect of 5% glucose on PKA activity in the cytosol fraction of RPMCs. The PKA activity was decreased in the cytosol fraction of RPMCs stimulated with 5% glucose (70.5% ± 11.5% of control, $p < 0.01$).

Figure 2(B) shows the effect of 5% glucose on PKA activity in the crude membrane fraction of RPMCs. The PKA activity was increased in the crude membrane fraction of RPMCs stimulated with 5% glucose (143.9% ± 52.9% of control, $p < 0.01$).

Figure 2(C) shows the effect of 5% glucose on PKA activity in the pure membrane fraction of RPMCs. The PKA activity did not change in the pure plasma membrane fraction of RPMCs stimulated with 5% glucose (108.6% ± 7.0% of control, p = nonsignificant).

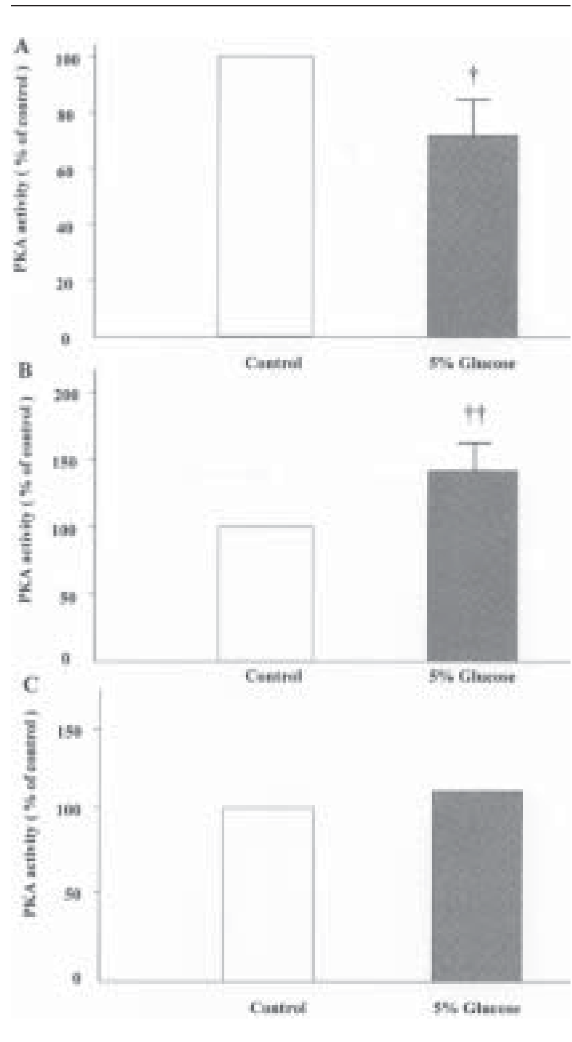

FIGURE 2 The effect of 5% glucose on protein kinase A (PKA) activity in the cytosol, crude membrane, and pure membrane fractions of rat peritoneal mesothelial cells. (A) The PKA activity was significantly decreased by 5% glucose stimuli in the cytosol fraction. † $p < 0.01$ as compared with control ($n = 8$ for each group). (B) The PKA activity was significantly increased by 5% glucose stimuli in the crude membrane fraction. †† $p < 0.01$ as compared with control ($n = 8$ for each group). (C) No significant change of PKA activity occurred in the pure membrane fraction. Nonsignificant as compared with control ($n = 4$ for each group).

Discussion

Recently, numerous studies have reported on the biologic molecular aspects of water permeability. Wade *et al.* (12) proposed a "membrane shuttle hypothesis" of water channel translocation in the principal cell of the cortical collecting duct. On the other hand, AQP1 is thought to be constitutively present in the plasma membrane and to work as a "housekeeping" type of water channel in the proximal tubule and descending limb of the Henle loop (13,14). Recently, however, it was proved that AQP1 translocation is promoted by secretin irritation in cholangiocytes (15). In addition, transient internalization of AQP1 by hyperosmotic stimuli in rat cardiac myocytes was reported (16).

In peritoneal mesothelial cells, little is known about the mechanism of short-term regulation of AQP1 protein. In PD, the osmolar gradient provided by glucose removes water from ESRD patients by ultrafiltration. And because ultrafiltration volume is correlated with glucose density of dialysate just after infusion, AQP1 translocation is more likely than protein synthesis to be the cause of increased water permeability under hyperosmotic condition in RPMCs. Patil *et al.* (17) reported that 8-bromo-cAMP or forskolin caused a significant increase in membrane permeability to water in *Xenopus* oocytes expressing AQP1, which suggests that stimulation of AQP1 activity may involve a cAMP-dependent mechanism. In addition, Han *et al.* (18) reported that AQP1 in rat kidney could be phosphorylated by a catalytic subunit of PKA, and that the amount of AQP1 protein was increased by a cAMP analog in *Xenopus* oocytes. Those findings reveal the possibility that PKA plays some role in the translocation of AQP1. We therefore examined the effect of a cAMP analog and a PKA

inhibitor on AQP1 translocation from cytosol to plasma membrane caused by hyperosmotic stimuli in RPMCs. Our results strongly suggest that PKA participated in the AQP1 translocation induced by hyperosmotic stimuli in RPMCs.

Two signal transduction pathways are generally listed for phosphorylation of a target protein via a PKA-dependent pathway. In the first pathway, PKA activated by cAMP moves into the nucleus as a second messenger and phosphorylates a gene adaptation protein, activating genetic transcription. In the second pathway, PKA phosphorylates serine or threonine residue (or both), activating the target protein. In the present experiment, we think that PKA is phosphorylating serine and threonine residues of AQP1, because the increase in the amount of AQP1 protein occurred too quickly to indicate protein synthesis. Hayakawa *et al.* (19) reported the manifestation of AQP1 mRNA a short time after hyperosmotic stimulation in a rat model of PD.

To confirm our hypothesis as described here, we examined the effect of 5% glucose on PKA activity separately in the cytosol fraction, crude membrane fraction, and pure membrane fraction of RPMCs. The PKA activity was significantly lower in the cytosol fraction of 5% glucose–stimulated RPMCs and significantly higher in the crude membrane fraction. However, PKA activity was not different in the pure plasma membrane fraction.

In NG108-15 cells, which are derived from nerve cells, PKA activity was localized in the cytosol around the nucleus (20). No report mentions localization of PKA in RPMCs. Our results suggest that the potency of the changes of PKA activity in each fraction are reflected in translocation of PKA from the cytosol fraction to the crude membrane fraction. The crude membrane fraction includes plasmalemma, Golgi bodies, mitochondria, lysosomes, ribosomes, and endosomes, but the pure membrane fraction includes only plasmalemma. In fact, we thought that activated PKA was translocated from cytosol to another cell organelle.

Conclusion

Given our findings, we hypothesize that hyperosmotic stimuli augment the PKA-dependent translocation of AQP1 to the plasma membrane, mediated by PKA translocation to the intracellular AQP1 store (Figure 3). Control of ultrafiltration in PD is vital for high-quality dialysis. The results of our study provide clinically important information about regulation of ultrafiltration in PD.

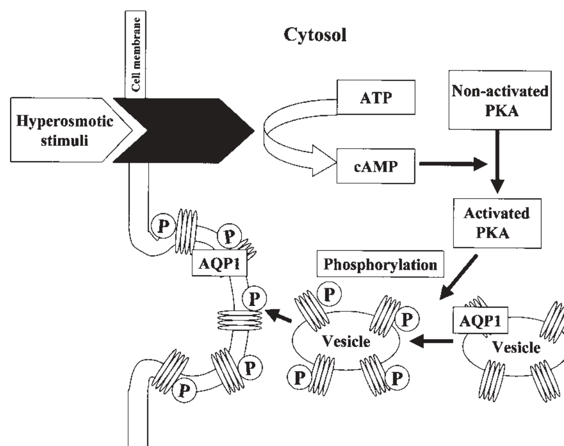

FIGURE 3 A hypothesized mechanism of aquaporin-1 (AQP1) translocation by hyperosmotic stimuli. ATP = adenosine triphosphate; PKA = protein kinase A; cAMP = cyclic adenosine monophosphate.

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