

Corticosteroids Induce Expression of Aquaporin-1 and Increase Transcellular Water Transport in Rat Peritoneum

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Abstract. The water channel aquaporin-1 (AQP1) is the molecular counterpart of the ultrasmall pore responsible for transcellular water permeability during peritoneal dialysis (PD). This water permeability accounts for up to 50% of ultrafiltration (UF) during a hypertonic dwell, and its loss can be a major clinical problem for PD patients. By analogy with the lung, the hypothesis was tested that corticosteroids may increase AQP1 expression in the peritoneal membrane (PM) and improve water permeability and UF in rats. First, the expression and distribution of the glucocorticoid receptor (GR) in the PM and capillary endothelium was documented. Time-course and dose-response analyses showed that a daily IM injection of dexamethasone (1 or 4 mg/kg) for 5 d induced an approximately twofold increase in the expression of AQP1 at the mRNA and

protein levels. The GR antagonist RU-486 completely inhibited the dexamethasone effect. The functional counterpart of the increased AQP1 expression was a significant increase in sodium sieving and net UF across the PM, contrasting with a lack of effect on the osmotic gradient and permeability for small solutes. The latter observation reflected the lack of effect of corticosteroids on nitric oxide synthase (NOS) activity and endothelial NOS isoform expression in the PM. In conclusion, corticosteroids induce AQP1 expression in the capillary endothelium of the PM, which is reflected by increased transcellular water permeability and UF. These data emphasize the critical role of AQP1 during PD and suggest that pharmacologic regulation of AQP1 may provide a target for manipulating water permeability across the PM.

Peritoneal dialysis (PD) is an established treatment for end-stage renal disease, accounting for about 15% of the total number of patients on dialysis worldwide (1). Solute transport during PD is best explained by the three-pore model based on computer simulations (2). According to this model, the major transport barrier of the peritoneal membrane (PM) is the capillary endothelium, which contains ultrasmall pores responsible for the diffusion of water but not that of solutes. Such ultrasmall pores explain the dissociation between sodium and water transport during the first hour of a hypertonic dwell, when the dialysate-over-plasma (D/P) ratio of sodium falls markedly (“sodium sieving”) as a result of free water diffusion into the peritoneal cavity (3). The fact that 50% of the ultrafiltration (UF) during a hypertonic dwell occurs through the ultrasmall pores illustrates their major clinical importance in PD patients (3).

The identification of the aquaporins, a family of integral plasma membrane proteins expressed in water-permeable tis-

sues, provided new insights in the molecular mechanisms involved in transcellular water transport (4). In addition to its abundant expression in the kidney, aquaporin-1 (AQP1) has been located in the endothelial cells lining nonfenestrated capillaries in a variety of tissues including the PM (5–7). The atomic structure of AQP1, a channel that facilitates the rapid transport of water across plasma membranes (8) and its distribution in the capillary endothelium have suggested that AQP1 is the molecular counterpart of the ultrasmall pore of the PM (9). The latter hypothesis has been strengthened by the loss of osmotically driven water transport across the PM in AQP1 knockout mice (10).

Although acute peritonitis remains the leading complication of PD, the loss of UF has now become the most frequent cause of technical failure in long-term PD patients (11). The loss of UF has severe clinical consequences, *e.g.*, increased extracellular fluid volume, and is associated with higher morbidity and mortality in PD patients (12). An increased absorption of glucose due to increased effective peritoneal surface area, with an early dissipation of the osmotic gradient, constitutes the most common cause of UF failure (13). Other causes include a reduced number of all types of pores with a marked decrease in the permeability for small solutes; increased lymphatic absorption; and a selective reduction of the transcellular water transport (3,14). The exact frequency and the mechanisms of UF failure due to reduced transcellular water transport remain unclear. Furthermore, despite the evidence supporting a role of

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AQP1, there has been no attempt to investigate the potential benefits of modulating its expression in the PM.

Aquaporin-1 is present in the endothelium lining the peribronchial vascular plexus in the lung, where it plays a significant role in vascular permeability (15). The expression of AQP1 in the capillary endothelium is induced by corticosteroids in perinatal rat lung, a process that participates in the rapid reabsorption of water from the distal lung in preparation for alveolar gas exchange (16). This induction occurs at the transcription level, because glucocorticoid response elements (GRE) have been identified in the promoter region of the mouse *Aqp1* gene (17). By analogy with the lung, the purpose of this study was to investigate the molecular and functional modifications induced by corticosteroids in the PM and particularly to test the hypothesis that the PM responds to high-dose corticosteroids by increasing in parallel the expression of AQP1 and the transcellular water permeability and UF.

Materials and Methods

Laboratory Animals

Studies were performed in male Wistar rats (Iffa Credo, Brussels, Belgium), aged 12 to 14 wk and weighing 250 to 350 g. Rats matched for age were randomly assigned to receive daily intramuscular injections of dexamethasone (Acidexam, Organon, Brussels, Belgium) or saline (0.9% NaCl, sham groups). For the dose-response protocol, rats were injected for 5 d with saline ($n = 18$) or dexamethasone 0.04 mg/kg ($n = 6$); 1 mg/kg ($n = 8$); and 4 mg/kg ($n = 8$). For the time-course protocol, rats ($n = 32$) were injected with saline or dexamethasone (4 mg/kg) for 1 d ($n = 10$); 3 d ($n = 10$); and 5 d ($n = 12$). All animals had access to standard chow and tap water *ad libitum*. In separate experiments, one group of rats ($n = 6$) was injected intramuscularly with dexamethasone (1mg/kg) and SC with the glucocorticoid antagonist RU-486 (Mifepristone, 70 mg/kg; Sigma-Aldrich, Bornem, Belgium) dissolved in mineral oil for 5 d. Three rats in this group died on the third day, whereas one rat underwent the whole treatment and the remaining two animals were treated with a dose of RU-486 administered every second day. A second group of rats ($n = 5$) was injected SC with mineral oil and IM with saline as sham for RU-486 and dexamethasone treatment. The experiments were conducted in agreement with local prescriptions and the NIH Guide for the Care and Use of Animals.

Peritoneal Permeability and Tissue Sampling

At day 6, sham and dexamethasone-treated rats ($n = 6$ in each group) were anesthetized with SC Nembutal (Sanofi, Brussels, Belgium) to perform a 2-h PD exchange with 15 ml of 7% glucose dialysate (Dianeal; Baxter, Nivelles, Belgium) as described previously (18). The 7% glucose concentration was selected for optimal assessment of the free-water permeability. Sodium sieving was defined as the decrease in D/P ratio for sodium during the first 30 min of the dwell (18). BP was monitored via a transducer implanted in the carotid artery as described (19). Transport of low molecular weight solutes was assessed by the D/P ratio of osmolality during the dwell, and the mass transfer coefficient (MTAC) for urea. Plasma and dialysate samples were collected at 0, 30, 60, and 120 min of dwell time. Urea, creatinine, hematocrit, glucose, sodium, total protein, and osmolality were assayed by standard methods (18), and prealbumin levels by nephelometry (19). White blood cells (WBC) were counted in a Bürker chamber, and dialysate cultures were obtained (18). Serum or plasma levels of corticosterone were measured by radio-immuno-

assay (ICN, Brussels, Belgium) in at least four rats from each group. At the end of the dwell, animals were sacrificed by exsanguination and samples from the visceral and parietal peritoneum were processed for fixation and mRNA/protein extraction as described previously (18). Samples for light microscopy were fixed in 4% paraformaldehyde in 0.1M phosphate buffer, pH 7.4, and embedded in paraffin. Samples for electron microscopy (EM) were fixed in 0.1 M phosphate buffer, pH 7.4 containing 0.1% glutaraldehyde and 4% paraformaldehyde, and embedded in Unicryl.

Antibodies

Immunostaining and Western blotting analyses were performed using affinity-purified rabbit antibodies against AQP1 (Chemicon International, Temecula, CA) or the glucocorticoid receptor (GR) (Santa Cruz Biotechnology, Santa Cruz, CA); monoclonal antibodies against eNOS and iNOS (Santa Cruz Biotechnology); and a monoclonal antibody against β -actin (Sigma, St. Louis, MO). Positive immunoblot controls included lysates from rat kidney and lung (AQP1); bovine aortic endothelial cells (eNOS); and mouse macrophages (iNOS).

Protein Extractions

Membrane and cytosolic extracts were prepared from visceral peritoneum, kidney, and lung as described previously (7,18). Briefly, tissues were homogenized in a Potter homogenizer on ice in a buffer containing 0.25 M sucrose, 20 mM imidazole (pH 7.4), 1 mM EDTA, and Complete protease inhibitor (Roche Diagnostics, Brussels, Belgium), followed by a brief sonication. The homogenates were centrifuged 15 min at $1000 \times g$ to remove nuclei and mitochondria. The supernatant was then centrifuged 2 h at $100,000 \times g$ to produce a pellet enriched for both plasma membrane and intracellular vesicles. The supernatant was considered as the cytosolic fraction. Protein concentrations were measured with the bicinchonic acid assay (Pierce, Erembodegem-Aalst, Belgium) using bovine serum albumin as standard. The samples were stored at -80°C until use.

Western Blot Analyses

SDS-PAGE and immunoblotting were performed as described previously (18,19). Samples of membrane fractions (5 to 15 μg) from kidney, visceral peritoneum, and lung were run on 12% gels. Efficiency of transfer to nitrocellulose was systematically tested by Ponceau red (Sigma) staining and β -actin immunoreactivity. After blocking, membranes were incubated with the primary antibody at 4°C overnight, washed, incubated for 1 h at room temperature with the appropriate peroxidase-labeled secondary antibodies (Dako, Glostrup, Denmark), and visualized with enhanced chemiluminescence (Amersham, Little Chalfont, UK). The immunoblot studies were performed at least in duplicate. Specificity was determined by incubation with non-immune IgG (Vector Laboratories, Burlingame, CA) or with the Anti-GR antibody pre-adsorbed with the cognate GR peptide (Santa Cruz Biotechnology). Densitometry analyses were performed with a studioStar Scanner (Agfa-Gevaert, Mortsels, Belgium) using the NIH-Image V1-57 software. The relative optical densities (in %, relative to controls) were obtained in duplicate.

Immunohistochemistry and Electron Microscopy

Immunostaining was performed on 6- μm -thick sections from the visceral and parietal peritoneum (18). After blocking in 0.3% H_2O_2 and incubation with 10% normal serum, sections were incubated successively for 45 min each with rabbit anti-AQP1 (1:200 dilution) or rabbit anti-GR antibody (1:50 dilution), biotinylated IgG (Vector),

Table 1. Clinical and biological parameters

Group ^a	n	BW day 1 (g)	BW day 6 (g)	Δ BW day 6 to day 1 (%)	Plasma Sodium (mEq/L)	Plasma Glucose (mg/dl)	Plasma Osmolality (mOsm/kg)	Plasma Urea (mg/dl)	Plasma Creatinine (mg/dl)	Plasma Corticosterone (nM)
Sham	6	341 ± 4	358 ± 4	+5.0 ± 1	148.3 ± 1	149.3 ± 11.5 ^b	299 ± 3	40.2 ± 1.7	ND	930 ± 50
Dexa 0.04 mg	6	344 ± 4	331 ± 4 ^c	-3.6 ± 1	148.8 ± 1	135.2 ± 21.1 ^b	296 ± 2	43.5 ± 2	ND	594 ± 29 ^c
Sham	8	247 ± 2	265 ± 3	+7.5 ± 1	146.4 ± 0.6	110.6 ± 3	302 ± 2	34.9 ± 1.6	0.28 ± 0.01	574 ± 50
Dexa 1 mg	8	243 ± 2	216 ± 4 ^c	-11.0 ± 1	146.1 ± 0.5	97.6 ± 2.5	305 ± 2	32.5 ± 1.4	0.27 ± 0.01	151 ± 16 ^c
Sham	4	258 ± 4	267 ± 5	+3.7 ± 1	144.5 ± 0.5	98.3 ± 3.5	299 ± 2	34.8 ± 1.8	0.37 ± 0.05	813 ± 39
Dexa 4 mg	8	258 ± 4	228 ± 5 ^c	-11.5 ± 2	145.0 ± 0.5	108.7 ± 4.0	303 ± 1	34.4 ± 1.5	0.34 ± 0.03	142 ± 23 ^c

^a Rats (matched for age) were treated with dexamethasone (Dexa, in mg/kg) or 0.9% NaCl (Sham) for 5 d (day 1 to day 5). Plasma parameters were obtained at time of sacrifice. ND, not done; BW, body weight.

^b Plasma glucose measured by glucooxidase versus hexokinase in other samples.

^c $P < 0.05$ versus sham.

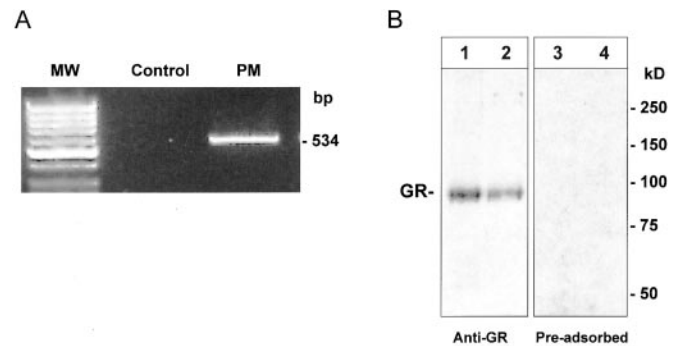


Figure 1. Expression of the glucocorticoid receptor (GR) in rat peritoneum. (A) Ethidium bromide stained 2% agarose gel showing RT-PCR product of GR at the expected size (534 bp). The amplification was performed using water (Control) or peritoneal cDNA (PM). MW: 100-bp ladder. (B) Western blot analyses of GR expression. Two samples (40 µg protein/lane) of cytosolic fraction extracted from rat visceral peritoneum were run on 7.5% SDS-PAGE and blotted on nitrocellulose. Identical strips were incubated with the affinity-purified anti-GR antibody (lanes 1 and 2) or the anti-GR antibody pre-adsorbed with the cognate GR peptide (lanes 3 and 4). The 95-kD band corresponding to GR is specifically detected. The films were exposed for 5 s (anti-GR antibody) and 2 min (pre-adsorbed antibody).

and avidin-biotin peroxidase (Vector). Immunolabeling was visualized using aminoethylcarbazole (Vector). Sections were viewed under a Leica DMR coupled to a Leica MPS60 photomicrographic system (Leica, Heerbrugg, Switzerland). The specificity of the immunolabeling was confirmed by incubation without primary antibody, with non-immune IgG (Dako), and with pre-adsorbed anti-GR antibody. For electron microscopy (EM) gold labeling, small pieces of visceral peritoneum embedded in Unicryl were cut in 70-nm-thick sections using a Reichert-S ultramicrotome (Leica) and collected on formvar-coated EM grids. The sections were preincubated in buffer-T containing 0.1% BSA, 0.05% Tween-20 in 100 mM Tris-HCl, pH 7.5, for 30 min. Sections were then incubated for 2 h with the anti-AQP1 primary antibody diluted in buffer-T, washed 6 × 5 min in buffer-T and incubated in a 1:20 dilution of 10 nm of gold-conjugated anti-rabbit antibodies (Amersham) in buffer-T for 45 min. Sections were washed 6 × 5 min, then stained for 3 min in 5% uranyl acetate followed by lead-citrate for 1 min and dried. Micrographs were taken in a Philips 400 EM at 13,000 final magnification. The EM gold labeling of endothelial cells was quantified on representative micrograph sections taken randomly from three different animals in each experimental condition. The total number of gold particles in endothelial cells was counted manually on each micrograph section and the endothelial length was measured. Results were expressed in particles per micrometer of endothelial length. The mean values were obtained from 11 to 16 micrographs in each group.

NOS Activity Assay

NOS enzymatic activities were measured using the L-citrulline assay as described previously in detail (18,19). Assays were performed for 30 min at 37°C. Assays were performed with or without Ca^{2+} to measure total versus Ca^{2+} -independent NOS activities, and calculate Ca^{2+} -dependent NOS activity. Determinations were performed in duplicate on four samples randomly selected in each group.

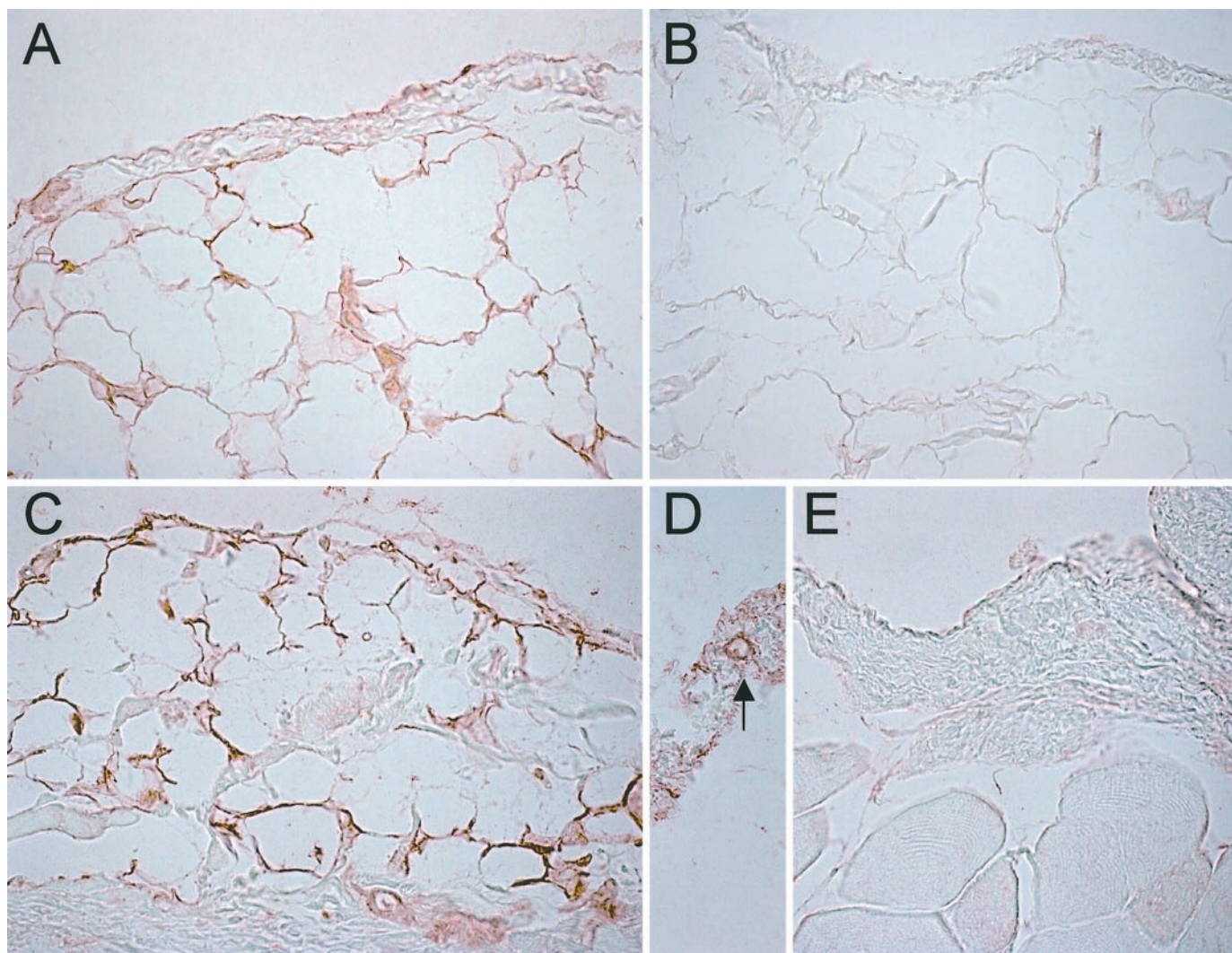


Figure 2. Distribution of GR in the rat peritoneum: immunostaining. A diffuse, intracellular staining for the GR is observed in the visceral (A and D) and parietal (C) peritoneum, distributed in adipocytes, mesothelial cells, and endothelial cells lining peritoneal capillaries (D, arrow). No specific staining is observed when similar sections from the visceral (B) and parietal (E) peritoneum are incubated with the anti-GR antibody pre-adsorbed with the cognate GR peptide. Magnification, $\times 250$.

RT-PCR and Semiquantitative Real-Time RT-PCR

Samples of visceral peritoneum were homogenized in TRIzol (Invitrogen, Merelbeke, Belgium), and total RNA was extracted according to the manufacturer's instructions. mRNA was isolated from total RNA using Dynabeads oligo(dt)₂₅ (DynaL Biotech, Compiègne, France) and reverse-transcribed into cDNA using SuperScript II Reverse Transcriptase (RT) (Invitrogen).

The primers used for the amplification of the GR were: GR SENSE 5'-tcgacgactgaaatgggcaa-3', GR ANTI-SENSE: 5'-gggaattcaatactcatg-gtc-3'. PCR conditions were as follows. Two microliters of RT sample were used in a total 50 μ l containing 2 mM MgSO₄, 0.2 mM each of dNTP, 0.2 mM each of forward and reverse primers and 1 unit of Platinum Taq DNA Polymerase High Fidelity (Invitrogen). Samples were submitted to a first denaturation for 4 min at 94°C then incubated during 30 cycles: denaturation at 94°C for 90 s; annealing at 54°C for 90 s, elongation at 72°C for 90 s, and final extension at 72°C for 8 min. The predicted length of the resulting PCR fragment was 534 bp. The PCR product was size-fractionated on 1.5% agarose gel, stained with ethidium bromide, purified by QIAquick Gel Extraction Kit

(QIAGEN Genomics Inc.) and subsequently sequenced by the GENOME express (Grenoble, France).

Changes in AQP1 mRNA levels were determined by semi-quantitative real time RT-PCR (iCycler IQ System, Bio-Rad) using SYBR green I detection of single PCR product accumulation. Primers for amplification of AQP1 and β -actin cDNA were designed using "Beacon designer 2.0" (Premier Biosoft International, CA) and were as follows: AQP1 SENSE: 5'gctgtcatgtatatcatgcg-cag 3', AQP1 ANTI-SENSE: 5'aggctatttcggccaagtgtg 3'; β -actin SENSE 5'gggttacgcgctccctcatg 3', β -actin ANTI-SENSE: 5'ccagctcggcaggatcttc 3'. The predicted lengths of the resulting PCR fragments were 107 bp (AQP1) and 90 bp (β -actin). The PCR products were size-fractionated and sequenced as described above. Real-time semi-quantitative PCR analyses were performed in duplicate with 200 nM of both sense and anti-sense primers in a final volume of 25 μ l using 1 unit of Platinum Taq DNA polymerase High Fidelity, 3 mM MgSO₄, 400 μ M dNTP, and SYBR Green I (Molecular Probe, Leiden, The Netherlands) diluted 1/10⁵. The PCR mix contained 10 nM fluorescein for initial well-to-well

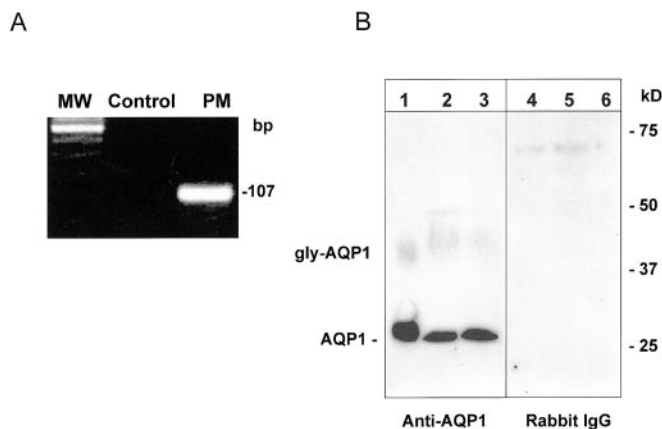


Figure 3. Expression of aquaporin-1 (AQP1) in rat normal peritoneum. (A) Ethidium bromide stained 2% agarose gel showing RT-PCR product of AQP1 at the expected size (107 bp). The amplification was performed using water (Control) or peritoneal cDNA (PM). MW: 100-bp ladder. (B) Western blot analysis of AQP1 expression. Membrane extracts from rat kidney cortex (lanes 1 and 4; 5 µg protein per lane) and visceral peritoneum (lanes 2, 3, 5, and 6; 15 µg protein/lane) were run on 12% SDS-PAGE and transferred to nitrocellulose. Identical strips were probed either with affinity-purified antibody against AQP1 (lanes 1 to 3) or non-immune, rabbit IgG at the same dilution (lanes 4 to 6). The major band at 28 kD corresponds to unglycosylated AQP1 (AQP1) and the more diffuse bands of between 35 and 50 kD corresponds to glycosylated AQP1 (gly-AQP1). The migration pattern of gly-AQP1 is slightly different in peritoneum versus kidney samples, probably reflecting the lower abundance of AQP1 in the peritoneum. Nonspecific bands of between 50 and 70 kD were detected with non-immune IgG. The films were exposed for 2 min.

fluorescence normalization. PCR conditions were 94°C for 3 min followed by 40 cycles of 30 s at 95°C and 1 min at 60°C. The melting temperature of the PCR product was verified at the end of each PCR by recording the increase in SYBR green fluorescence upon slow renaturation of DNA (initial denaturation at 98°C for 1 min followed by stepwise decrease of the temperature by 10 s steps of 0.5°C). To exclude amplification from contaminating genomic DNA, samples of mRNA that had not been reverse-transcribed were run in parallel PCR reaction; these controls always remained negative. For each assay, standard curves were prepared by serial fourfold dilutions of cDNA of the sham rats. For each sample, the ΔC_t ($C_t\text{AQP1} - C_t\beta\text{-actin}$) was calculated. The relative changes in AQP1/ β -actin mRNA ratio between sham and dexamethasone-treated rat samples were determined by the formula $2^{-\Delta\Delta C_t}$, where $\Delta\Delta C_t$ is the difference in ΔC_t between dexamethasone-treated and sham rats.

Data Analyses

Data are presented as mean $\pm$ SEM. Comparisons between results from different groups were performed using *t* test or one-way ANOVA, as appropriate. Statistical significance was defined as $P < 0.05$.

Results

Clinical and Biologic Parameters

The clinical and biologic parameters of the rats treated for 5 d with various doses of corticosteroids or saline (sham) are

shown in Table 1. In comparison with sham rats, administration of dexamethasone was reflected by a significant weight loss and suppression of endogenous corticosterone in all groups. At time of sacrifice, sham and dexamethasone-treated rats were similar in terms of prealbumin levels, plasma sodium, glucose, osmolality, urea and creatinine levels (Table 1). None of the rats included in the sham or dexamethasone-treated groups died during the study. None of the rats treated for 5 d showed peritonitis, as demonstrated by a clear dialysate at the end of the dwell, low dialysate WBC counts, and negative dialysate cultures (data not shown).

Expression and Distribution of the Glucocorticoid Receptor in the Peritoneum

The expression of the GR in rat peritoneum was documented by RT-PCR, which showed the expected 534-bp product (Figure 1A). The identity of the PCR product was confirmed by sequence analysis (data not shown). The expression of the GR at the protein level was demonstrated by Western blot (Figure 1B), which identified a strong immunoreactive band at 95 kD corresponding to the molecular mass of GR. No band was identified when the blot was probed with the anti-GR antibody pre-adsorbed with the cognate peptide. The distribution of GR in the PM was investigated by immunostaining (Figure 2). The intracellular GR was detected in the visceral and parietal peritoneum, where its distribution included mesothelial cells, adipocytes, and endothelial cells lining peritoneal capillaries. The staining pattern was abolished when incubation was performed with the pre-adsorbed anti-GR antibody (Figure 2).

Expression of AQP1 in the Peritoneum: Influence of Corticosteroids

The expression of AQP1 in the rat peritoneum was verified by detecting the expected 107-bp product by RT-PCR (Figure 3A) and subsequent sequence analysis (data not shown). Immunoblot analysis with the affinity-purified antibody against AQP1 identified the core (AQP1: 28 kD) and glycosylated AQP1 (Gly-AQP1: 35 to 50 kD) in rat kidney (positive control) and visceral peritoneum samples (Figure 3B).

Time-course analysis (Figure 4A) demonstrated that high-dose dexamethasone (4 mg/kg) induced a progressive increase in the expression of AQP1 in the peritoneum. Treatment with dexamethasone for 1 and 3 d did not significantly influence AQP1 expression at the protein level, despite suppression of endogenous corticosterone (sham [337 ± 39 nmol] versus 1 d [14 ± 2 nmol] versus 3 d [9 ± 2 nmol]; $n = 4$; $P < 0.01$). In contrast, administration of dexamethasone for 5 d was reflected by a significant increase of AQP1 expression in the peritoneum and, as expected, suppression of endogenous corticosterone (sham [337 ± 39 nmol] versus dexamethasone [7 ± 1 nmol]; $n = 4$; $P < 0.01$).

The dose-response of a 5-d administration of dexamethasone on AQP1 expression in the rat peritoneum was also investigated (Figure 4B). Administration of low-dose and intermediate-dose dexamethasone (0.04 mg/kg and 0.4 mg/kg) was sufficient to decrease endogenous corticosterone

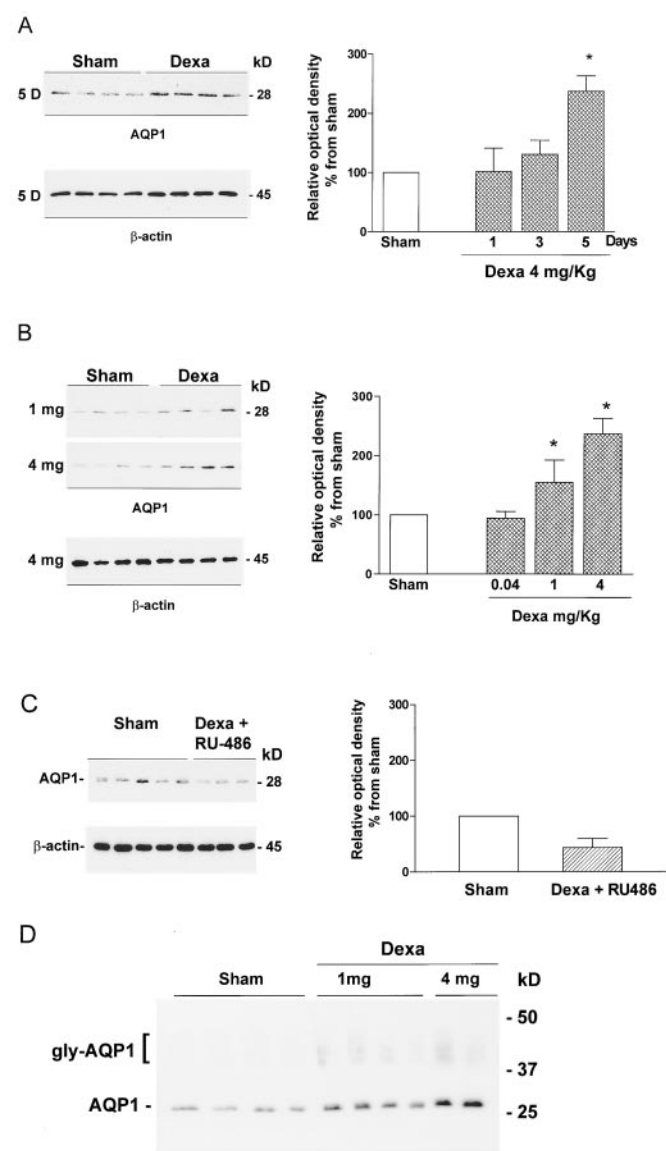


Figure 4. Effects of corticosteroids on AQP1 expression in the peritoneum. (A) Time-course. Representative immunoblot for AQP1 (28 kD) in the visceral peritoneum of rats treated with saline (sham) or high-dose (4 mg/kg) dexamethasone for 5 d (5D). Samples (10 μ g protein/lane) were run in 12% SDS-PAGE, transferred to nitrocellulose and probed with the affinity-purified antibody against AQP1. The blot was stripped and reprobed using a monoclonal antibody against β -actin (45 kD). Similar blots were obtained after 1 day and 3 d of treatment, and the relative optical densities for AQP1 (normalized against those obtained in the appropriate age-matched sham rats) are shown in the right panel. As compared with sham, the signal intensity for AQP1 increased between days 1 and 3, becoming significant at day 5 (1.02 $\pm$ 0.3-fold, n = 4; 1.31 $\pm$ 0.24-fold, n = 4; 2.37 $\pm$ 0.26-fold, n = 6; respectively). (B) Dose-dependence. Representative immunoblots for AQP1 (28 kD) in the visceral peritoneum of rats treated for 5 d with saline (sham) or dexamethasone 1 mg/kg and 4 mg/kg. The latter blot was stripped and reprobed for β -actin. The densitometry values shown in the right panel are normalized against those obtained in the appropriate age-matched sham rats. In comparison with sham, the signal intensity for AQP1 is not modified with low-dose dexamethasone (0.04 mg/kg: 0.94 $\pm$ 0.11-fold, n = 6, data not shown) but significantly increases in rats treated with high-dose

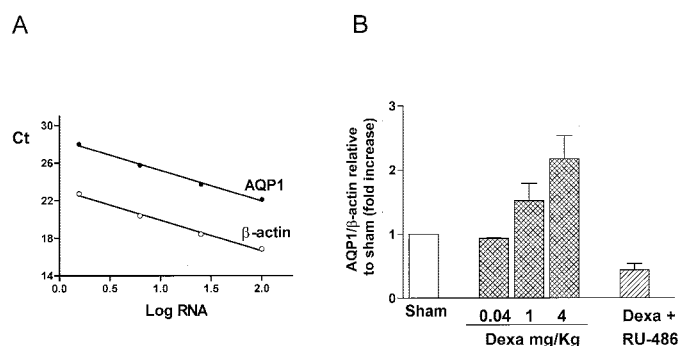


Figure 5. Effect of corticosteroids on AQP1 mRNA in the peritoneum: semi-quantitative real-time RT-PCR. (A) Standard curves of amplification for AQP1 and β -actin in peritoneum: the slopes (mean result of four independent experiments) were obtained by linear regression, and they are similar for β -actin (-3.25) and AQP1 (-3.27). Threshold cycle (Ct) is defined as the number of cycles at which the fluorescence signal reached a fixed threshold above baseline. (B) Quantification of AQP1 mRNA in the peritoneum of rats treated with various doses of dexamethasone, expressed as the relative increase over sham (dexamethasone 0.04 mg/kg: 0.93 $\pm$ 0.1-fold; 1 mg/kg: 1.5 $\pm$ 0.3-fold; 4 mg/kg: 2.2 $\pm$ 0.4-fold). This parameter was calculated using the $2^{-\Delta\Delta Ct}$ formula after normalization to β -actin (ΔCt) and determination of the difference in ΔCt ($\Delta\Delta Ct$) between dexamethasone-treated and sham rats. The cotreatment of dexamethasone 1 mg/kg and RU-486 abolished the effect of dexamethasone alone and decreased the amount of AQP1 mRNA (sham versus dexamethasone + RU-486, 0.44 $\pm$ 0.15-fold, n = 3). It must be noted that the samples are identical to those used for immunoblotting (Figure 4C).

(Table 1), but not to alter the expression of AQP1 in the peritoneum (data not shown). In contrast, treatment with high-dose dexamethasone (1 mg/kg and 4 mg/kg), reflected by the suppression of corticosterone (Table 1), induced the expression of AQP1 (approximately twofold at 4 mg/kg) in the peritoneum (Figure 4B).

The induction of AQP1 expression by 1 mg/kg dexamethasone was totally abolished when the antagonist RU-486 was administered in the same animals (Figure 4C). As previously reported (16), the expression of AQP1 in the lung was also induced by high-dose dexamethasone (1 and 4 mg/kg; Figure

dexamethasone for 5 d (1 mg/kg: 1.55 $\pm$ 0.38-fold, n = 8; 4 mg/kg: 2.37 $\pm$ 0.26-fold, n = 8; * P < 0.05 versus sham). (C) The administration of RU-486 to rats treated with high-dose dexamethasone (1 mg/kg) prevents the increase in AQP1 expression in the visceral peritoneum (sham versus dexamethasone + RU-486, 0.44 $\pm$ 0.16-fold, n = 3). It must be noted that a single rat survived to the daily injection of RU-486 + dexamethasone, while two received RU-486 on alternate days + dexamethasone daily (see text). The immunoblot was performed as in panel B. (D) As compared with sham, administration of high-dose dexamethasone for 5 d strongly induces the expression of AQP1 in rat lung (1 mg/kg: 1.60 $\pm$ 0.11-fold; 4 mg/kg: 2.62 $\pm$ 0.27-fold, both in relative optical density). The immunoblot was performed as in panel B.

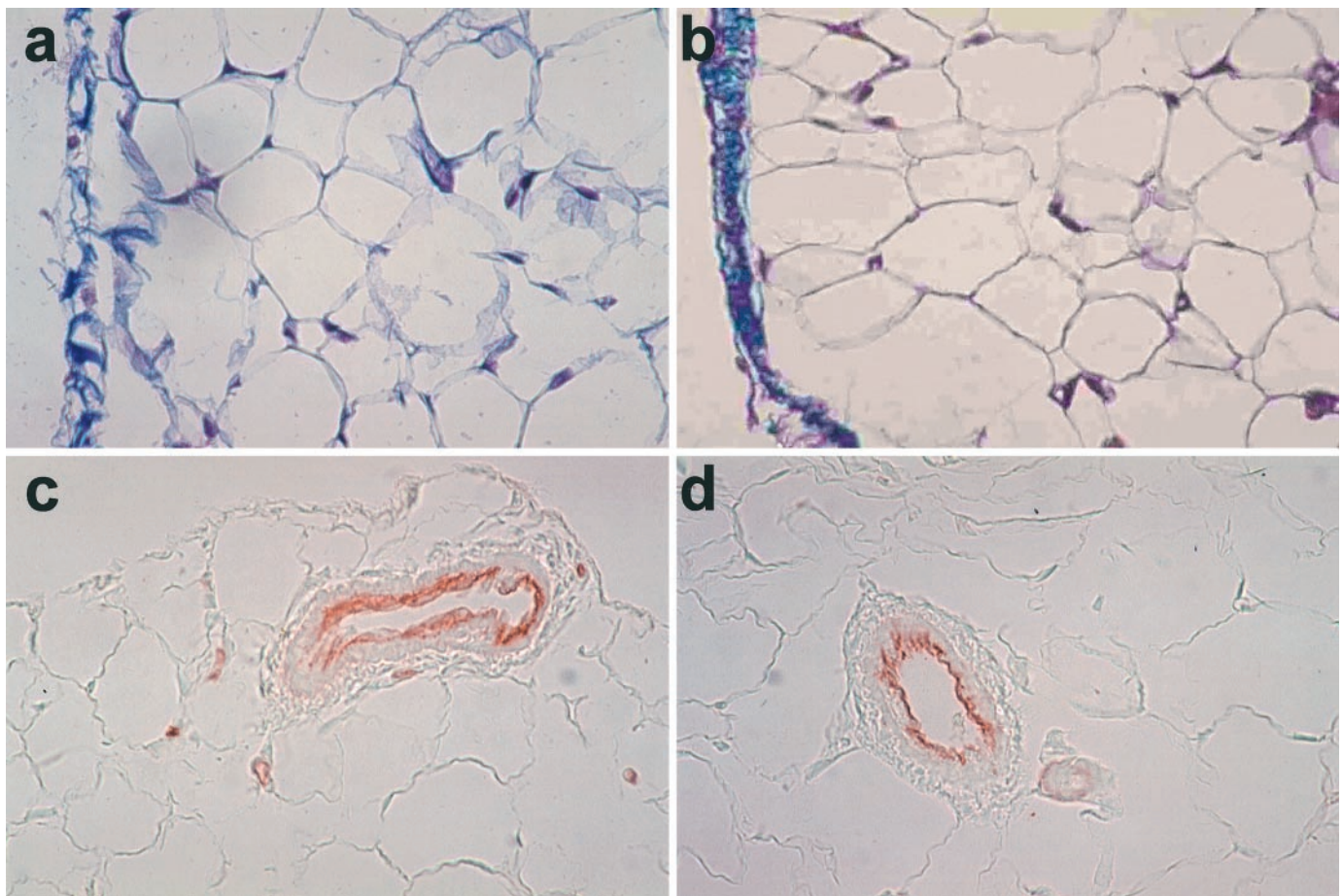


Figure 6. Corticosteroids treatment on the peritoneum: morphology and immunostaining for AQP1. Trichrome-blue staining (a and b) and immunostaining for AQP1 (c and d) in visceral peritoneum from sham rats (a and c) and rats treated with high-dose (4 mg/kg) dexamethasone (b and d) for 5 d. As compared with sham (a) the general morphology of the visceral peritoneum is preserved in dexamethasone-treated rats (b). Immunostaining shows that the distribution of AQP1 in the endothelium lining small venules in the peritoneum is similar in sham (c) and dexamethasone-treated rats (d). Magnification: $\times 270$.

4D), whereas the expression of AQP1 in the kidney remained unchanged (data not shown).

The effect of corticosteroids on AQP1 expression in the peritoneum was substantiated by real-time PCR analysis performed on rat visceral peritoneum samples (Figure 5). As compared with sham animals, AQP1 mRNA expression was not increased by treatment with low-dose (0.04 mg/kg) dexamethasone but increased progressively in rats treated with 1 mg/kg (1.5 ± 0.3 -fold) and 4 mg/kg (2.2 ± 0.4 -fold) dexamethasone. The increase in AQP1 mRNA induced by 1mg/kg dexamethasone was totally abolished by concomitant administration of RU-486 (Figure 5B).

Effect of Corticosteroids on the Distribution of AQP1 in the Peritoneum

The administration of high-dose dexamethasone did not induce structural abnormalities in the peritoneum and, at the light microscope level, was not reflected by changes in the pattern of AQP1 distribution in the endothelium lining peritoneal vessels and capillaries (Figure 6). The effect of high-dose dexamethasone on the ultrastructural distribution

of AQP1 in endothelial cells lining peritoneal capillaries was therefore investigated using EM gold labeling. Representative micrographs shown in Figure 7 demonstrate that AQP1 was detected primarily along plasma membranes of endothelial cells lining peritoneal capillaries. AQP1 was also detected in red blood cells in the lumen (L) of the endothelium (not shown), but it was absent from other cells such as mesothelial cells (Figure 7B). In comparison with sham rats (Figure 7B), labeling for AQP1 was markedly increased in samples from rats with high-dose dexamethasone (Figures 7 C and D). In tissues from dexamethasone-treated animals, where AQP1 labeling was most abundant, AQP1 labeling sometimes appeared intracellular (Figures 7, C and D). However, this might be due to the large infoldings of plasma membranes between endothelial cells. No labeling was observed in the absence of affinity-purified anti-AQP1 antibodies (Figure 7A). Quantitative analysis of the number of AQP1 gold particles per micrometer of endothelial cell length (Table 2) confirmed the significant twofold increase in gold labeling for AQP1 in high-dose dexamethasone-treated rats with respect to sham.

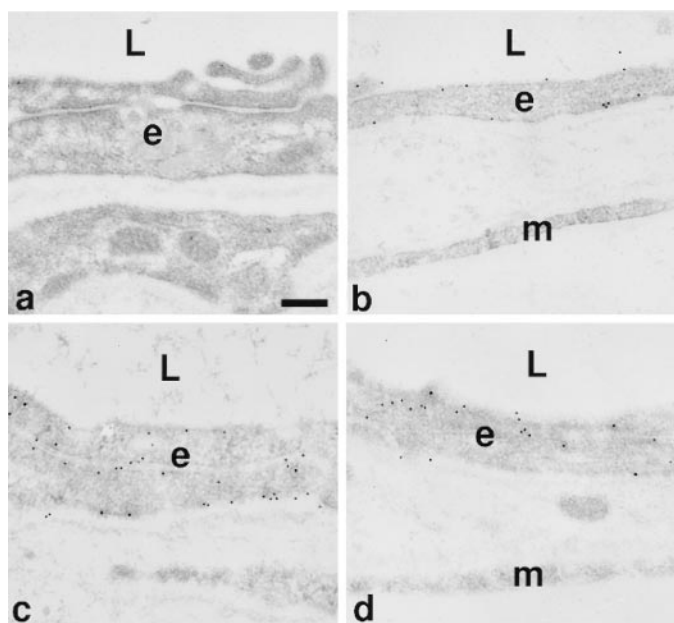


Figure 7. Immunoelectron microscopic localization of AQP1 in the peritoneum. Ultrathin sections from the visceral peritoneum of sham rats (b) or rats treated with high-dose dexamethasone (1 mg/kg: a and c; 4 mg/kg: d) for 5 d were incubated with (b and d) or without the affinity-purified anti-AQP1 antibody (a) and visualized with goat anti-rabbit IgG conjugated to 10-nm colloidal gold particles. (a) No labeling is observed in the absence of affinity-purified anti-AQP1 antibody. (b) AQP1 labeling is seen in both apical and basal plasma membranes of endothelial cells lining peritoneal capillaries in sham rats. (c and d) The abundance of AQP1 in endothelial cells is markedly increased in dexamethasone-treated animals. No labeling for AQP1 is observed in other cell types (m, mesothelial cells), except the red blood cells (not shown), within the lumen (L) of capillaries. Scale bar: 250 nm.

Effect of Corticosteroids on Water and Small Solutes Permeability across the Peritoneum

A 2-h PD exchange was performed at day 6, to evaluate the functional counterpart of the 5-d treatment with dexamethasone or saline. BP levels measured at the beginning of the dwell were similar in sham and high-dose dexamethasone-treated rats (sham [119 ± 4 mmHg] *versus* 1 mg/kg dexamethasone [118 ± 4 mmHg]; sham [106 ± 9 mmHg] *versus* 4 mg/kg dexamethasone [123 ± 4 mmHg]). The net UF (Volume OUT – volume IN, in ml/kg) remained unchanged in rats

treated with low-dose (0.04 mg/kg) dexamethasone but significantly increased ($+44 \pm 11\%$ increase) in rats treated with high-dose (1 mg/kg) dexamethasone (Table 3). Similarly, the sodium sieving remained unchanged in rats treated with low-dose dexamethasone but significantly increased ($+31 \pm 8\%$ increase) in rats treated with high-dose dexamethasone (Table 3, Figure 8A). The effect on sodium sieving was observed despite similar osmotic gradients in all groups (Figure 8B). It must be noted that MTAC for urea (Table 3), as well as total protein loss in the dialysate (not shown) were similar in sham *versus* high-dose dexamethasone rats. These data demonstrate that treatment with high-dose dexamethasone improves UF and increases transcellular water permeability of the PM without affecting the osmotic gradient between dialysate and plasma or the peritoneal permeability for small solutes.

Corticosteroids Do Not Influence Endothelial NOS Activity and Expression in the Peritoneum

To rule out a potential effect of high-dose dexamethasone on eNOS expression and/or activity in the peritoneum, we measured total NOS activity in the visceral peritoneum of sham *versus* rats treated with dexamethasone (1 mg/kg) for 5 d. The total NOS activity (sham [0.13 ± 0.02 pmol citrulline/mg protein per min] *versus* dexamethasone [0.14 ± 0.2 pmol citrulline/mg protein per min; $n = 4$] and Ca^{2+} -dependent NOS activity (sham [0.12 ± 0.03 pmol citrulline/mg protein per min] *versus* dexamethasone [0.13 ± 0.02 pmol citrulline/mg protein per min]; $n = 4$) were similar in both groups (Figure 9A). The very low level of Ca^{2+} -independent NOS activity confirmed the lack of infection/inflammation in the peritoneum of these animals, as also demonstrated by the lack of iNOS signal on immunoblots (data not shown). Further immunoblot analyses confirmed that the expression of eNOS (140 kD) in the peritoneum was not influenced by high-dose (1 and 4 mg/kg) dexamethasone (Figure 9B).

Discussion

The water channel AQP1 is considered as the ultrasmall pore of the PM, but it remains unknown whether its pharmacologic modulation might influence water permeability and improve UF during PD. Our studies revealed that administration of corticosteroids increases the expression of AQP1 in the peritoneum at the mRNA and protein levels. The effect of dexamethasone is completely inhibited by the GR antagonist RU-

Table 2. Quantification of immunogold labeling for AQP1 in endothelial cells of the peritoneum^a

Parameter/Group	Sham ($n = 11$)	Dexa 1 mg ($n = 12$)	Dexa 4 mg ($n = 16$)
AQP1 gold particles/ μm (mean $\pm$ SEM)	3.66 ± 0.42	7.42 ± 1.34^b	7.20 ± 1.23^c
Endothelial cell length (total length in μm)	97	75	86

^a The rats were treated with saline (sham) or high-dose dexamethasone (1 or 4 mg/kg) for 5 d. n represents the number of different micrographs analyzed from three rats in each group.

^b $P = 0.017$ *versus* sham.

^c $P = 0.03$ *versus* sham.

Table 3. Effects of corticosteroids on peritoneal permeability parameters: water and small solutes^a

Group	n	Net Ultrafiltration (ml/kg BW)	Sodium Sieving (% decrease T ₃₀ to T ₀)	MTAC Urea (ml/min)
Sham	6	39.2 ± 1	13.9 ± 0.4	0.18 ± 0.02
Dexa 0.04 mg	6	40.8 ± 2	13.9 ± 0.8	0.16 ± 0.02
Sham	6	38.3 ± 3	14.5 ± 0.9	0.18 ± 0.03
Dexa 1 mg	6	52.2 ± 1 ^b	18.6 ± 0.7 ^b	0.15 ± 0.02

^a Rats were treated with saline (sham), low-dose (0.04 mg/kg), or high-dose (1 mg/kg) dexamethasone for 5 d. BW, body weight.

^b $P < 0.01$.

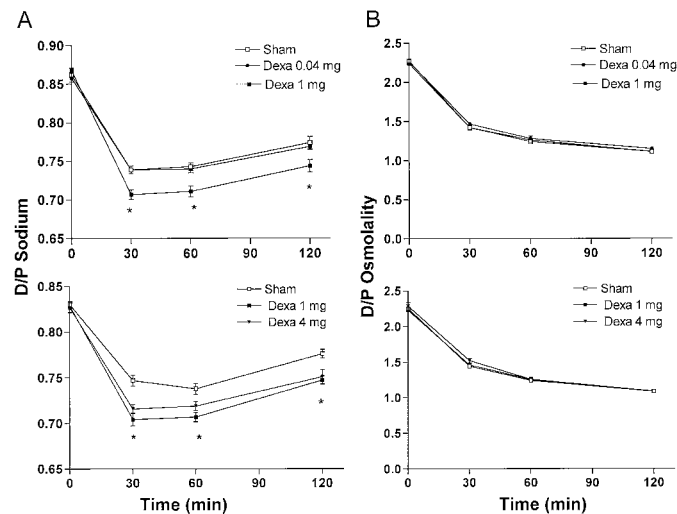


Figure 8. Effects of corticosteroids on peritoneal permeability parameters. Rats were treated with saline (sham) or dexamethasone at 0.04 mg/kg (Dexa 0.04), 1 mg/kg (Dexa 1 mg) or at 4 mg/kg (Dexa 4 mg) for 5 d and a 2-h PD exchange was performed on day 6. Dialysate-to-plasma (D/P) ratios of sodium (A) and osmolality (B) in sham (□), Dexa 0.04 mg (●), Dexa 1 mg (■), and Dexa 4 mg (▼) during the 2-h dwell are shown. A significant increase (A) in sodium sieving (*i.e.*, an increased drop in D/P sodium at 30 min) is observed in rats treated with high-dose dexamethasone, whereas the osmolality gradient between the dialysate and plasma is similar in all groups (B). There were 6 rats in each group.

486. The increase in AQP1 expression is mirrored by a significant increase in transcellular water transport (monitored by sodium sieving) and net UF in absence of any significant effect on the osmotic gradient and permeability for small solutes.

The 5-d administration of corticosteroids in rats is followed by a parallel increase in the expression of AQP1, the sodium sieving, and the UF across the PM. Furthermore, our immunoelectron microscopy data show that AQP1 is expressed on both luminal and basolateral membranes of endothelial cells lining peritoneal capillaries and that the amount of AQP1 gold particles in these cells is significantly increased by corticosteroids. The functional effects of the induction of AQP1 in the PM, which represent the counterpart of the decreased water transport observed in the AQP1 knockout mice (10), support the role of AQP1 in mediating transcellular water transport in

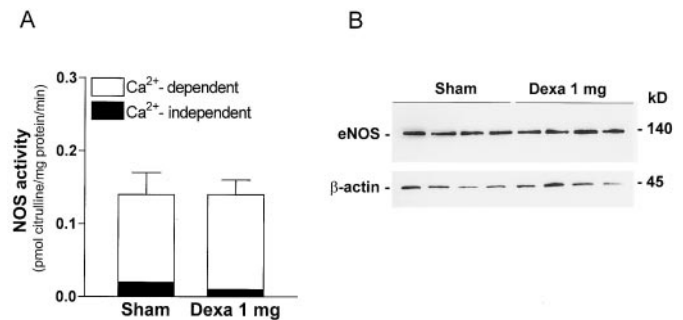


Figure 9. Effect of corticosteroids on NOS activity (A) and eNOS expression (B) in the visceral peritoneum. (A) Dexamethasone administration (1mg/kg for 5 d) does not modify the total and Ca²⁺-dependent NOS activity (L-citrulline assay, $n = 4$ in each group) in the rat peritoneum. (B) As compared with sham rats, treatment with high-dose dexamethasone (1 mg/kg for 5 d) does not influence eNOS expression (Western blot) in the visceral peritoneum. Samples (15 μ g protein/lane) were run on 7% SDS-PAGE and probed with the monoclonal antibody against eNOS (140 kD). The gel was stripped and reprobed with a monoclonal antibody against β -actin. The eNOS film was exposed for 5 min.

the PM and its importance for net UF. Aquaporin-1 molecules form homotetramers in the plasma membrane, with each 28-kD subunit containing an independent water pore (20). Recent studies have revealed the atomic structure of AQP1, which explains both its selectivity for water and its ability to facilitate rapid water transport across membranes (8). The 2.8 Å diameter of the narrowest part of the AQP1 pore is close to the diameter of a water molecule, and it contains a site formed by the aromatic side chains of Phe56 and His180 and the positively charged Arg195 that provides selectivity against ions and protons (21). The structure of AQP1 also fits the postulated ultrasmall pore size (*i.e.*, less than 6 Å in diameter) necessary to explain the effectiveness of glucose as an osmotic agent in PD.

In contrast to AQP1, the role of other aquaporins in the PM remains controversial. AQP3 and AQP4 have been detected in the PM by RT-PCR (22). However, AQP3 and AQP4 are not induced by corticosteroids (23), and subsequent studies failed to document a significant expression of both proteins in the capillary endothelium of the PM (10,18). The fact that AQP3 and AQP4 are expressed in erythrocytes (24) and muscle fibers (25), respectively, may explain cross-contamination in PM

samples. Furthermore, the HgCl₂-insensitivity of AQP4 (6), as well as the fact that the osmotic water permeability across the PM is unchanged in AQP4 knockout mice (10) render unlikely that AQP4 contributes significantly to water permeability across the PM.

The effect of corticosteroids on AQP1 expression is tissue-specific and dose- and time-dependent (Figures 4 and 5). Our studies in the rat confirm the presence of GR in endothelial cells (26,27). Low-dose dexamethasone, sufficient to suppress endogenous corticosterone, had no significant effect on AQP1 expression, whereas high-dose dexamethasone induced a progressive increase in AQP1 expression in the PM that was significant after 5 d of treatment. The induction of AQP1 by glucocorticoids in peritoneal capillaries is explained by the presence of GRE in the promotor region of the gene coding for AQP1 (17). These cis-acting regulatory elements, which are conserved along species (17), are probably involved in the complex developmental pattern of AQP1 expression (16). The similar induction of AQP1 expression in the PM and lung suggests interesting structural and functional similarities between serosal membranes, because AQP1 is located in peribronchial vessels and visceral pleura and participates in lung water clearance and pleural fluid movement, respectively (15,16,28). It must be noted that *in vivo* administration of the GR antagonist RU-486, although complicated by a high mortality rate in this rat model, totally inhibited the effect of dexamethasone on AQP1 expression.

Pharmacologic doses of corticosteroids (1 to 4 mg/kg dexamethasone) were required to increase AQP1 expression and water permeability in the PM. This dose of corticosteroids is twice that used in rat lung (16), and it corresponds approximately to what is required to maximally induce GRE-mediated responses in rodents (27). Although one can argue whether such high-dose corticosteroids could be beneficial given their side effects, it is tempting to suggest that pharmacomodulation of AQP1 with glucocorticoids may offer therapeutic perspective for selected PD patients. This possibility should take advantage of the experience accumulated in patients with inflammatory diseases, as well as recent developments in the use of corticosteroids (29). Our results also suggest that the cumulative exposure of the PM to glucocorticoids, and perhaps individual sensitivity, may participate in the regulation of the basal permeability of the PM.

Previous studies have shown that corticosteroids regulate inflammation in the PM (30–32) and improve peritoneal sclerosis in animal models (33) and PD patients (34). The lack of inflammation and/or infection of the PM in our rat model — attested by negative dialysate cultures and lack of iNOS induction — renders unlikely that such antiinflammatory properties are involved in the functional modifications observed. On the other hand, the release of NO in endothelial cells lining peritoneal capillaries plays an important role in clinical situations complicated with UF failure, such as acute peritonitis or long-term PD (18,35). In these situations, the upregulation of eNOS parallels an increase in the effective peritoneal surface area, mediating a faster than normal reabsorption of small solutes and glucose, and a dissipation of the osmotic gradient

(36). The administration of dexamethasone in rats has been shown to modulate the expression of eNOS in a tissue-dependent fashion (37). Our data clearly show that, at the doses used, corticosteroids do not upregulate eNOS nor increase NOS activity in the PM (Figure 9). Accordingly, treatment with corticosteroids does not alter the transport of small solutes or the osmotic gradient across the PM (Figure 8). The effect on UF can thus be attributed to the increased density of AQP1 ultrasmall pores in the capillary endothelium.

In summary, corticosteroids induce AQP1 expression and water permeability in the PM, which is reflected by increased UF. These data emphasize the critical role of AQP1 during PD and show that pharmacologic regulation of AQP1 may ultimately provide a target for manipulating water permeability across the PM and treating some cases of UF failure in PD.

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