Hypothyroidism Increases Osmotic Water Permeability (P_f) in the Developing Renal Brush Border Membrane

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ABSTRACT

The osmotic water permeability (P_f) of the rabbit proximal tubule brush border membrane vesicles (BBMV) increases during maturation and is mediated by an increase in aquaporin-1 (AQP1) protein expression. Serum thyroid hormone levels increase after birth and have been shown to play a role in the maturation of other renal transport functions. We examined the hypothesis that thyroid hormone plays a role in the maturational increase in osmotic water permeability. Hypothyroidism was induced by addition of 0.1% propylthiouracil (PTU) to the drinking water of pregnant rabbits (starting 9 d before delivery) and was continued until the rabbits were studied as adults (9–11 wk). Some animals received thyroid hormone replacement by daily injection with triiodothyronine (T₃; 10 µg/100 g body weight) for three days before study. P_f was found to be higher in BBMV from hypothyroid (82.7 ± 5.5 µm/s) than from euthyroid (60.6 ± 4.0 µm/s) and T₃-replacement rabbits (69.0 ± 5.0 µm/s) (*p* < 0.05). The activation energy (E_a; in kcal/deg·mol) of P_f was not different among the three experimental groups (euthyroid 5.6 ± 0.9, hypothyroid 4.9 ± 0.8, T₃-replacement 5.0 ± 1.0; *p* = NS), nor was the percentage mercury inhibition of P_f (euthyroid 66.5 ± 5.3, hypothyroid 74.2 ± 3.2 and T₃-replacement 73.1 ± 4.3; *p* = NS). AQP1 expression, measured by immunoblotting,

was highest in BBMV from hypothyroid rabbits (*p* < 0.05). Membrane fluidity, measured as steady-state generalized polarization (GP) of Laurdan, which is inversely related to membrane fluidity, was significantly different between the three groups (GP: euthyroid 0.307 ± 0.004, hypothyroid 0.271 ± 0.004 and T₃-replacement 0.287 ± 0.003; for all *p* < 0.05). These data demonstrate that the maturational increase in thyroid hormone levels is not responsible for the maturational increase in water transport. Surprisingly, congenital hypothyroidism in rabbits is associated with an increased P_f when rabbits are studied as adults. The higher P_f in hypothyroid adult rabbits is due to a higher expression of AQP1 protein as well as a greater membrane fluidity than in euthyroid rabbits. (*Pediatr Res* 53: 1001–1007, 2003)

Abbreviations

AQP1, aquaporin 1
BBMV, brush border membrane vesicles
PTU, propylthiouracil
GP, generalized polarization
P_f, osmotic water permeability
E_a, activation energy

The proximal tubule reabsorbs approximately two-thirds of the glomerular ultrafiltrate (1). This occurs in a nearly iso-osmotic manner due to the high water permeability of this segment. We have previously demonstrated that the osmotic water permeability of both apical (brush border) and basolateral membranes increased during postnatal development (2, 3). In both membranes, this was accompanied by an increase in

aquaporin-1 (AQP1) expression; the predominant water channel in proximal tubular membranes (4).

The factors that are responsible for the maturational change in proximal tubule water transport and AQP 1 expression remain unknown. Thyroid hormone levels rise postnatally (5, 6) and have been shown to be a factor in developmental changes in proximal tubular function, including NaCl transport (7), Na⁺/H⁺-antiporter activity (5) and phosphate transport (6). The role of thyroid hormone with regard to maturation of water transport in the proximal tubule remains unknown.

The aim of the present study was to investigate the effect of thyroid hormone on the development of proximal tubule water transport. Our hypothesis was that the postnatal increase in thyroid hormone is necessary for the increase in proximal tubule AQP1 expression. We examined the effects of chemi-

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cally induced hypothyroidism on the osmotic water permeability (P_f) of rabbit renal BBMV functionally and at the level of protein abundance. We also studied the effect of hypothyroidism on another important factor in water permeability of BBMV, namely membrane fluidity (8).

METHODS

Animals. Pregnant New Zealand White rabbits were housed at our institution for at least 9 d before delivery. The protocol for these studies was approved by the Institutional Animal Care and Research Advisory Committee. They were kept on a 12 h-12 h light-cycle, at an ambient temperature of 16–22°C and were fed standard laboratory chow. Pups were cared for by their mother until weaned and were studied at 9–11 wk of age. Hypothyroidism was induced in pups by treatment with propylthiouracil (PTU), which prevents the postnatal increase in serum thyroid hormone levels (thyroxine) (7). Treatment was initiated 9 d before birth by adding 0.1% PTU to the drinking water of pregnant does and was continued until study (adulthood, 9–11 wk). Thyroid replacement (“ T_3 -replacement”) therapy was given with daily s.c. injections with 3,5,3'-triiodothyronine (T_3 , 10 μ g/100 g body weight) for 3 d including 2 h before sacrifice as previously described in our laboratory (7). Age matched control pups and their mothers had access to tap water.

Materials. Chemicals were purchased from Sigma Chemical Co.-Aldrich (St. Louis, MO, U.S.A.) and Fluka BioChemika (Buchs, Germany). 6-Dodecanoyl-2-dimethylaminonaphthalene (Laurdan) was obtained from Molecular Probes (Eugene, OR, U.S.A.). Anti-AQP1 antibody was purchased from Alomone Labs (Jerusalem, Israel), anti- β -actin from Sigma Chemical Co.-Aldrich and horseradish peroxidase-conjugated anti-rabbit MAb from Amersham (Arlington Heights, IL, U.S.A.). Solution osmolalities were measured by freezing point depression (Advanced Instruments, Norwood, MA, U.S.A.; osmometer model 3D3).

Serum thyroid hormones. Blood was collected at time of sacrifice and centrifuged at 1500 rpm for 10 min. Serum was frozen at -80°C. Serum concentrations of triiodothyronine (T_3) and thyroxine (T_4) were assessed with radioimmunoassays (Diagnostic Systems Laboratories, Webster, TX, U.S.A.).

Brush border membrane vesicle preparation. Brush border membrane vesicles were prepared as described previously in our laboratory (9, 2). Briefly, kidneys were removed promptly and immediately placed in ice-cold PBS (137 mM NaCl, 2.7 mM KCl, 10.1 mM Na_2HPO_4 , 1.7 mM KH_2PO_4 , pH 7.4). Kidneys from 3–5 neonates were pooled. The capsule was removed and the cortex was dissected. After mincing, the cortex was transferred into isolation buffer (300 mM D-mannitol, 16 mM HEPES, 5 mM EGTA, adjusted to pH 7.5 with Tris-HCl; protease inhibitors aprotinin (2 μ g/mL), leupeptin (2 μ g/mL) and phenylmethylsulfonyl fluoride (175 μ g/mL)). Tissue was homogenized by 15 strokes with a Potter homogenizer, after which cell precipitation was induced using 230 μ L of 1.0 M $MgCl_2$. The homogenate was shaken vigorously for 10 s every 5 min for 20 min. Subsequently, the homogenate was centrifuged at $2500 \times g$ for 15 min at 4°C. The supernatant was

aspirated, added to 230 μ L 1.0 M $MgCl_2$ and shaken vigorously for 10 s every 5 min for 20 min, then centrifuged for 15 min at $2500 \times g$ at 4°C. The supernatant was then centrifuged at $48,400 \times g$ for 30 min at 4°C. The pellets were resuspended in 1.5 mL ice-cold resuspension buffer (5.0 mM HEPES, pH 7.4; osmolality adjusted to 80 mosmol with d-mannitol). The protein content was determined in the crude homogenate and BBMV using BCA protein assay (Pierce, Rockford, IL). Alkaline phosphatase activity was determined to assess enrichment, as previously described (9, 2). There was no difference in enrichment between control, hypothyroid and T_3 -replacement animals (fold increase, respectively 6.0 ± 0.4 , 7.0 ± 1.0 and 6.1 ± 0.8 , $p = NS$).

Stopped-flow kinetics. BBMV were brought to a concentration of 0.6 mg protein/mL. BBMV (100 μ L) were rapidly mixed with 240 mosmol resuspension buffer (100 μ L) using a stopped-flow apparatus (SFM-3, Biologic, France). These solutions are routinely used in measuring water permeability and have previously been used in our laboratory (2, 3, and 8). The cuvet was illuminated using a 75W-xenon arc lamp and a monochromator that was set at 400 nm. Light scattering was measured using a photomultiplier tube (Biologic, France) oriented at a 90° angle with regard to the illumination axis. Data were collected at 10 ms intervals for 2 s using Bio-kin software (Biologic, France). Five raw tracings were averaged and analyzed subsequently. P_f was calculated as previously described (3). Briefly, the osmotic water permeability was calculated from the stop-flow light scattering data as described by Van Heeswijk *et al.* (10). The scattered light intensity was normalized to an initial value of 1, then fit to a double exponential curve:

$$I(t) = b - c_1 e^{-k_1 t} - c_2 e^{-k_2 t}$$

where $I(t)$ is the light intensity at time t , b is the asymptote, c_i are the coefficients and k_i are the rate constants. The mean rate constant was determined by the following equation:

$$\bar{k} = \frac{c_1 k_1 + c_2 k_2}{c_1 + c_2}.$$

P_f was then calculated from the following equation:

$$\bar{k} = \bar{V}_w P_f (A/V_0) C_m,$$

where $\bar{V}_w$ is the molar water volume, A/V_0 is the surface area to initial vesicle volume ratio and C_m is the osmolality of the extravascular solution. The initial surface area to volume ratio was calculated assuming that the vesicles were spheres. We had previously demonstrated that the diameters of the neonatal and adult BBMV is 200 nm that was used in the present calculations (2).

Temperature dependence and mercury-sensitivity. To examine the activation energy (E_a) of P_f , experiments were conducted at 20, 25, 33 and 40°C. E_a was calculated from the slope of the Arrhenius plot ($\ln k$ versus temperature⁻¹ (11, 12)):

$$E_a = -[\text{slope} \times R]$$

where R is the gas constant (1.987 kcal.deg-1.mol-1). The slope was determined using linear regression analysis. The temperature dependence of water transport gives valuable insight into the mechanism of water movement. Channel mediated water flow has a low activation energy while water movement through the lipid bilayer has a high activation energy (8, 12).

Inhibition of water transport across water channels was studied by incubating BBMVs with 0.5 mM HgCl_2 at room temperature ($\sim 23^\circ\text{C}$) for 10 min and measuring P_f at 20°C .

Immunoblotting for AQP1. Immunoblots were performed as described previously (2, 3). Briefly, BBMVs samples (40 μg total protein) were brought to a 50 μL volume using isolation buffer. After addition of 10 μL loading buffer, samples were heated to 95°C for 5 min, chilled on ice and loaded on a 10% SDS gel. Samples were transferred overnight onto PVDF membrane at 4°C .

Anti-AQP1 was used at 1:1,000 and anti- β -actin at 1:5,000 dilutions. After preincubation with Blotto, membranes were incubated with the primary antibody at room temperature for two hours, washed four times over 40 min, and incubated with the secondary antibody at room temperature for one hour. Detection was done using ECL kit (Amersham).

Membrane fluidity measurements. Membrane fluidity was assessed with the use of Laurdan, a fluorophore sensitive to the polarity of its environment (13). Penetration of water into the membrane causes a spectral shift and increased emission at 490 nm. Generalized polarization (GP) is used to quantify this shift from the following relation:

$$\text{GP}_{\text{laurdan}} = [\text{I}_{440} - \text{I}_{490}] / [\text{I}_{440} + \text{I}_{490}]$$

where I_{440} and I_{490} are the emission intensities at 440 nm and 490 nm, respectively (13).

BBMV (100 μg protein) were incubated with 0.33 nmol of Laurdan at 37°C for >20 min. Using a photon counting spectrofluorometer (PC1, ISS Inc., Urbana-Champaign, IL, U.S.A.), samples were excited at 340 nm. Emission intensities were measured at 440 nm and 490 nm. Experiments were conducted at 37°C . Generalized polarization was subsequently calculated using ISSPC software (ISS Inc.).

Statistical analysis. Data were analyzed using SigmaStat statistical software (Jandel Corporation, San Rafael, CA, U.S.A.). Data are presented as means ($\pm$ SEM). For measures of osmotic water permeability, each preparation was measured at each temperature so that the data were analyzed using two-way repeated measures ANOVA. Where the preparation was used once, the data were analyzed using Student's t -tests and one-way ANOVA, according to the design of the experiment.

RESULTS

Serum thyroid levels. Serum levels of thyroid hormone are shown in Table 1. The suppression of thyroid function in PTU-treated animals was evident from a significantly lower serum concentration of T_4 in both the hypothyroid and T_3 -replacement groups. In hypothyroid animals, the levels of the active compound T_3 were reduced by approximately one-third

Table 1. Serum thyroid hormone levels

Group (N)	T_3 (ng/dL)	T_4 ($\mu\text{g}/\text{dL}$)
Euthyroid (10)	178 ± 18	4.2 ± 0.4
Hypothyroid (9)	$120 \pm 12^\dagger$	$1.5 \pm 0.1^\ddagger$
T_3 -replacement (10)	$>1000^\ddagger\#$	$1.5 \pm 0.1^\ddagger$

$^\dagger p < 0.05$ vs. euthyroid.

$^\ddagger p < 0.001$ vs. euthyroid.

$\# p < 0.001$ vs. hypothyroid.

compared with control, euthyroid animals. T_3 -replacement was confirmed to be effective by the high levels of T_3 measured 2 h after the last dose of T_3 .

Osmotic water permeability. Representative tracings for the three experimental groups are shown in Fig. 1. At 20°C , vesicles from hypothyroid animals had a faster rate of change in BBMVs volume, measured as a change in light scattering due to an imposed osmotic gradient, compared with controls. T_3 -replacement for three days resulted in rates that were comparable to controls. Calculation of P_f showed similar results at all four temperatures studied (Fig. 2). The results showed that congenital hypothyroidism was associated with a significant increase in P_f ($p < 0.05$ versus euthyroid). This effect was fully reversed by T_3 -replacement ($p = \text{NS}$ versus euthyroid and $p < 0.05$ versus hypothyroid).

Activation energy. The activation energy of the osmotic water permeability of each experimental group was calculated from the slope of the Arrhenius plot (Fig. 3). The temperature dependence, and thus the E_a of the BBMVs P_f , was not different between the three groups.

Mercury inhibition. The addition of 0.5 mM HgCl_2 to BBMVs caused not only a change in rate-constant (k), but a decrease in signal amplitude as well. To take this observation into account, the degree of inhibition of P_f by HgCl_2 was calculated from the initial slope of the averaged tracings. The percentage inhibition was not different among the three groups (euthyroid 66.5 ± 5.3 , hypothyroid 74.2 ± 3.2 and T_3 -replacement 73.1 ± 4.3 ; $p = \text{NS}$).

Immunoblot

The protein abundance of AQP1 and β -actin is shown in Fig. 4. Densitometry showed that hypothyroidism significantly increased the expression of AQP1 in the renal brush border membrane (AQP1/ β -actin ratio: hypothyroid 3.27 ± 0.81 , control 0.66 ± 0.33 , T_3 -replacement 0.85 ± 0.36 ; $p < 0.05$). There was no difference between the control group and the animals that received T_3 replacement to correct their hypothyroidism.

Membrane fluidity

Membrane fluidity was measured to examine an aspect of water transport other than the expression of AQP1 that may contribute to the effects of hypothyroidism and T_3 -replacement on P_f (Fig. 5). Generalized polarization of Laurdan was significantly lower in hypothyroid than in euthyroid BBMVs ($p < 0.001$). This effect of hypothyroidism was only partially reversed by T_3 -treatment. Since a lower generalized polarization corresponds to a higher membrane fluidity, this indicates that

Figure 1. Typical tracings for measurement of P_f in euthyroid, hypothyroid and T_3 -replacement (dotted line) BBMVs at 20°C. BBMVs were prepared with 80 mosmol resuspension buffer and rapidly mixed with an equal volume of 240 mosmol resuspension buffer, creating an inward osmotic gradient of 80 mosmol. Vesicle shrinkage increased light scattering. Averaged tracings were normalized to initial fluorescence and fitted to a double-exponential curve.

hypothyroidism renders the BBM more fluid and that short-term treatment with T_3 -replacement decreases the fluidity promptly, although not to control levels.

DISCUSSION

This study examined the effects of congenital hypothyroidism on maturation of P_f in BBMVs. In BBMVs obtained from rabbits with chemically induced congenital hypothy-

roidism, P_f was higher than in BBMVs from euthyroid animals. This effect was reversed by replacement of T_3 for 3 d. E_a and mercury inhibition of P_f were not different among the three groups. Hypothyroidism was also associated with a higher AQP1 protein abundance and membrane fluidity, which were partially reversed by T_3 -replacement, indicating that the effects are mediated by thyroid hormone rather than by direct effects of PTU. The combined results

Figure 2. P_f for euthyroid, hypothyroid and T_3 -replacement groups at 20–40°C. Means were calculated using repeat measures ANOVA. Error bars denote SEM. The P_f of the hypothyroid BBMVs was significantly higher than the euthyroid controls and the T_3 -replacement groups.

show that thyroid hormone affects both channel-mediated water transport and transport through the lipid bilayer in BBMVs simultaneously.

Maturation of water transport in the proximal tubule is a complex process. The neonatal tubule has an osmotic water permeability that is higher than that of the adult segment, yet the apical and basolateral membranes of the neonatal tubule have lower water permeabilities than the adult tubules (14, 2, 3). The characteristics of the apical membrane water transport in the neonate include a lower P_f and AQP1 expression and a higher activation energy than the adult (2). The activation energy for water transport is thought to be determined by the membrane fluidity, which in turn is determined by the lipid and protein composition of the membrane (8).

Thyroid hormone concentrations in the newborn rabbit are significantly lower than the adult (15, 16). The level of thyroid hormone increases around the third week of life. We have previously shown that thyroid hormone affects both active and passive transport in the proximal tubule (7). We now demonstrate that thyroid hormone affects expression of AQP1. Because the adult expression of AQP1 is higher than the neonate, we expected to see a low expression level of AQP1 in the hypothyroid adult. However, we found the opposite. The hypothyroid animals had a higher expression of AQP1 and a higher osmotic water permeability. This was unexpected and is quite remarkable.

The effects of hypothyroidism on membrane fluidity were also remarkable. Previously, hypothyroidism was shown to not affect or even decrease fluidity of renal BBMVs (17, 18), but these data were obtained in adult rats, that had been euthyroid

before and were treated with PTU for only 3 wk. On the other hand, in neonatal rats, T_4 treatment decreased membrane fluidity of intestinal BBMVs in perinatal (<24 h) and 12-d-old rats. This suggests that thyroid hormone accelerates the maturational decrease in membrane fluidity (19). The fluidity data from the present study are consistent with this, since T_3 -replacement for 3 d decreased fluidity of BBMVs. Thus, our results suggest that thyroid hormone is required for the normal development of the lipid bilayer of the renal brush border membrane.

The effects of hypothyroidism on the P_f of the brush border membrane may also be the result of well organized adaptive mechanisms to effects hypothyroidism has on other proximal tubule functions or on functions of other segments of the nephron. In rabbits that are hypothyroid from birth, proximal straight tubule NaCl transport is reduced and is reversible by T_3 -replacement (7). With solute transport being limited in the hypothyroid animal, an increased P_f would ensure that fluid reabsorption was not limited as is the case with the AQP1 knockout mice (20). Hypothyroidism is also associated with changes in tubule length and diameter, which may lead to decreases in the brush border membrane surface area and thus require higher P_f to allow for sufficient reabsorption of water. The increased P_f of the brush border membrane may also reflect a greater fluid reabsorption in the proximal tubule that serves as a compensatory mechanism for impaired function of the more distal nephron segments. The effects of hypothyroidism on water transport may also play a role in the development of myxedema.

In conclusion, this study shows that congenital hypothyroidism in rabbits is associated with an increased P_f in renal BBMVs

Figure 3. Arrhenius plot of the temperature dependence of P_f . The activation energy (E_a) was calculated from the slope of the regression lines. E_a was not different among the three groups. Error bars denote SEM. The P_f of the hypothyroid BBMVs was significantly higher than the euthyroid controls and the T_3 -replacement groups.

Figure 4. Immunoblot of AQP1 protein abundance. Lanes were loaded with 40 μ g protein. Densitometry showed AQP1/ β -actin ratio: hypothyroid 3.27 ± 0.81 , control 0.66 ± 0.33 , T₃-replacement 0.85 ± 0.36 ; $p < 0.05$.

Figure 5. Generalized polarization (GP) of steady-state Laurdan fluorescence. BBMVs (100 μ g protein) were incubated with 0.33 nmol Laurdan at 37°C for 20 min before measurements. Excitation wavelength was 340 nm. GP was calculated from the emission intensities at 440 and 490 nm. Membrane fluidity is inversely related to GP, indicating that hypothyroidism results in a lower fluidity of BBMVs and that T₃-replacement for three days decreases fluidity, but not to euthyroid levels.

at 9 wk of age. This is due to a higher protein expression of AQP1 as well as a delay in the maturational decrease of membrane fluidity. These effects are readily reversible by T_3 -replacement. This suggests that thyroid hormone is not required for the maturational increase of P_f in BBMVs, although thyroid hormone may play a role in the normal development of the membrane lipid composition. The mechanism by which thyroid affects P_f and aquaporin 1 expression remains to be elucidated.

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