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Prolonged Ethanol Ingestion Increases Renal AQP2 and AQP3 Expression in Adult Rats and in Their Offspring

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Abstract. This study evaluates the effect of prolonged ethanol ingestion on the renal ability to concentrate urine. Suckling Wistar rats born to mothers given ethanol before and during gestation and suckling periods (ethanol-exposed offspring) were used and the results were compared with those obtained from offspring of dams given diets containing no ethanol. Comparisons were also made between progenitors with or without prolonged ethanol ingestion. Body and kidney weights; arginine-vasopressin (AVP) and aldosterone plasma levels; plasma, urine and renal papillary osmolality; urine outflow; kidney AQP2, AQP3 and AQP4 expression and diencephalon AVP mRNA expression were determined. As compared with control offspring, the ethanol-exposed offspring present i) lower body and kidney weights; ii) lower urine outflow; iii) higher renal AQP2 and AQP3 mRNA; iv) higher renal AQP2 protein content and v) higher urine and renal papillary osmolality. These changes were also observed in the ethanol-treated progenitors, although they were of smaller magnitude. Plasma osmolality, renal AQP4 mRNA, AVP plasma levels and diencephalon AVP mRNA expression were not affected by the ethanol treatment. Plasma levels of aldosterone were only significantly increased in the ethanol-exposed suckling rats. It is concluded that maternal ethanol ingestion before and during gestation and suckling periods affects the renal function of the offspring, up-regulating renal AQP2 expression by an AVP-independent mechanism. Ethanol-treated progenitors manifest similar renal changes, although of lesser magnitude than the offspring.

Key words: Ethanol — Kidney — AQP2 — AQP3 — AQP4 — AVP

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Introduction

In mammalian kidney most of the filtered water is reabsorbed through the water channel aquaporin 1 (AQP1), constitutively present in the apical and basolateral membranes of the proximal tubule and thin descending limb. Final urine is concentrated by water re-absorption along the collecting duct to the hypertonic medullar interstitium. The water permeability of the collecting duct is mediated by the apical membrane AQP2 and the basolateral membrane AQP3 and AQP4. Regulation of this water permeability depends on the capacity of arginine-vasopressin (AVP) to control, via an increase in cAMP levels, AQP2 expression and its targeting to the apical membrane of the collecting duct cells. Extensive studies have shown that alterations in these processes are involved in almost all situations where diuresis/antidiuresis occurs. Thus, conditions with impaired urine concentration ability have decreased renal expression of AQP2 mRNA, whereas increased levels of AQP2 expression and apical plasma membrane AQP2 targeting are seen under water retention, such as severe congestive heart failure, liver cirrhosis, pregnancy and syndrome of inappropriate antidiuretic hormone secretion (*see* Ward, Hammond & Harris, 1999; Nielsen et al., 2002 for reviews).

Prolonged ethanol ingestion results in altered water-electrolyte homeostasis and induces body dehydration (Wright & Donlon, 1979; Dow-Edwards et al., 1989; Magner et al., 1991; Carney, Gillies & Ray, 1995). However, the molecular bases of such derangements have not been addressed. The aim of the current work was to determine whether maternal ethanol consumption before and during gestation and suckling periods affects water-electrolyte homeostasis and AQP2 turnover in the offspring. The study was extended to the progenitors (mothers and fathers).

Materials and Methods

Materials

(α^{32}P)-UTP was purchased from Amersham Biosciences. Unless otherwise indicated, the other reagents used in the current study were obtained from Sigma Chemical, Madrid, Spain.

Animals and Ethanol Administration

Male and female Wistar rats weighing between 250 and 300 g were randomized into two groups and ethanol was administered with tap water to one of them as previously described (Tavares et al., 1999). Briefly, rats were mated after receiving successively ad libitum 5, 10, and 15% of ethanol in the drinking water during 3 weeks and a consumption of 20% ethanol was maintained for 4 additional weeks. The presence of sperm in the vaginal smear denoted the first day of pregnancy. Water containing 20% ethanol and food were given *ad libitum* to pregnant females during the pregnancy and suckling periods and to males. The day of parturition was designated as first day of lactation and day 21, the end of lactation period. During this period, the pups (ethanol-exposed offspring) had free access to the nipples. The group of animals that served as control received water and a standard rat chow diet (Panlab 04) *ad libitum* and were handled in the same way as the ethanol-treated animals. The rats were maintained under automatically controlled temperature (22–23°C) and 12 hour light-dark cycles, with lights on at 07:00 h.

Measurements of body and kidney weights, drinking water intake, urine and plasma collection were done between 9:00 h and 10:00 h to avoid changes due to circadian rhythms.

The animal care and research protocols were in accordance with the guidelines of the European convention.

Papillary Osmolality and Urine Parameters

Papillary osmolality was measured as described by Combet et al. (2001). Briefly, the rats were anesthetized by i.p. injection of urethane (14%) and following removal of the kidneys, the whole papilla was excised from each kidney, weighed, homogenized manually with a glass potter, after addition of 200 µl of distilled water, and centrifuged at 12,000× *g* during 1 min. The osmolality of the supernatant was measured with an osmometer (Osmometer Gonotec, Osmomat 030) and the osmolality of the papilla was calculated on the assumption that 80% of the wet weight is water.

Urine was collected at the end of the suckling period from either the progenitors during 24 h or during 1 h from the offspring. The offspring could not be separated from their parents for more than 1 hour because they were suckling.

Plasma Analysis

Blood samples were taken from the heart of urethane-anesthetized rats, collected on EDTA tubes and centrifuged immediately at 4°C. Plasma levels of AVP and aldosterone were measured by radioimmunoassay by Cerba Laboratory (Barcelona, Spain).

Tissue Preparation for Northern and Western Blot Assays

After the brain and kidneys were removed from urethane anesthetized rats, the entire kidney (suckling rats), the kidney medulla (adult rats) and the diencephalon were rinsed with ice-cold saline solution and immediately dropped in liquid nitrogen and frozen at −80°C until use. Kidneys of 21 days-old suckling rats were used, because in rats the capacity to concentrate urine develops during the first 3–4 wk of life (Rane et al., 1985; Yamamoto et al., 1997).

cDNA Cloning and Sequencing

An AVP cDNA fragment of 495-bp was generated from rat diencephalon by RT-PCR. 2 µg of poly(A)$^+$ RNA isolated from rat diencephalon were primed with a random primer using a SuperScript preamplification system kit (Life Technologies), as described by the manufacturer. The designed primers, based on the rat AVP cDNA sequence (Rehbein et al., 1986), were: sense, 5′-GATGCTCAACACTACGCTC-3′ and antisense 5′-TCAGTAGACCCGGGGCTTG-3′. The PCR profile was performed as described (Peral et al., 2002) and the AVP cDNA fragment was sequenced on both strands (3100 genetic Analizer automated sequencer).

RNA Preparation and Northern Blot Analysis

Total RNA was extracted from the kidney and diencephalon as described by Chomczynski and Sacchi (Chomczynski & Sacchi, 1987). AQP2, AQP3 and AQP4 antisense riboprobes were generated as before (Murillo-Carretero, Ilundain & Echevarría, 1999). (α^{32}P)-UTP-labelled AVP antisense riboprobe was synthesized using a plasmid DNA containing the sequence of AVP, which was linearized by Apa I and transcribed in vitro with SP6 RNA polymerase (Amersham). Northern hybridization was carried out using rat kidney Poly(A)$^+$, as reported (Murillo-Carretero et al., 1999). For a direct comparison of band intensities, equal amounts (10 µg) of mRNA from kidneys of ethanol-treated and -untreated rats were loaded into individual lanes of a single gel. Relative quantification of either AQP2, AQP3, AQP4 or AVP mRNA expression was determined by densitometry of the blots using PCBAS program version 2.0 (Raytest).

Western Blot Analysis

Apical membrane preparations were obtained from renal epithelial cells (Garcia-Delgado et al., 2001). Immunoblotting was performed according to Laemmli, as previously (Peral et al., 2002). AQP2 was detected with a polyclonal antibody (1:1000 dilution) raised against the COOH-terminal peptide sequences of rat AQP2 (Nielsen et al., 1993), generously provided by Søren Nielsen (University of Aarhus, Denmark). β-Actin has been used to normalize the AQP2 density values and, hence, the membranes were subsequently incubated with β-actin antiserum at 1:10,000 dilution.

The immunoreactive bands were viewed using the enhanced chemiluminescence procedure (Pierce). The relative abundance of the bands was quantified using PCBAS program version 2.0 (Raytest).

Statistical Analysis

Data are presented as mean ± SEM for *n* separate animals. In the figures, vertical bars represent the SEM. Comparison between different experimental groups was evaluated by the two-tailed Student's *t*-test.

Results

Effect of Ethanol on Body and Kidney Weights, and on Plasma Parameters

Maternal ethanol ingestion during gestation and lactation periods reduced the body (40% reduction)

Table 1. Renal and plasma parameters measured in control and ethanol-treated rats

	Offspring 21 days old		Progenitors 5 months old	
	Control	Ethanol	Control	Ethanol
Urine Outflow (μl/h)	131 ± 12	19* ± 5	920 ± 20	290* ± 10
Urine Osmolality (mosmol/Kg)	738 ± 50	2428* ± 145	621 ± 32	1694* ± 170
Papillary Osmolality (mosmol/Kg)	397 ± 4	447* ± 11	362 ± 15	458** ± 37
Plasma Osmolality (mosmol/Kg)	330 ± 4	320 ± 9	305 ± 5	314 ± 5
AVP (pg/ml)	6.7 ± 0.4	6.5 ± 0.9	6.7 ± 0.5	7.6 ± 0.33
Aldosterone (ng/L)	400 ± 45	540** ± 53	252 ± 21	312 ± 45

Urine and renal papillary osmolality, and plasma levels of AVP and aldosterone were measured as indicated in Methods. Values are means ± SEM of 6 to 10 rats.
*$p < 0.001$, **$p < 0.05$

and kidney (40% reduction) weights of their offspring. In the progenitors, ethanol ingestion slightly reduced (15% reduction) their body and kidney weights and decreased water consumption from 19.5 ± 2.6 ml/day to 13 ± 2 ml/day, $n=8$, $p < 0.05$.

Urine outflow, urine and renal papillary osmolality, as well as plasma levels of AVP and aldosterone, were measured in both, suckling rats (21-day-old) and in their progenitors (5-month-old rats). The results show (Table 1) that urine and renal papillary osmolality were significantly higher in ethanol-exposed rats (offspring and progenitors) than in ethanol-untreated animals. According to these observations, urine outflow was significantly decreased by ethanol: by 7-fold in the offspring and by 3.7-fold in the progenitors. Ethanol treatment significantly increased plasma aldosterone levels in the offspring, but not in the progenitors and had no significant effect on plasma levels of AVP.

Plasma osmolality was also measured, but it was not affected by ethanol treatment in any of the experimental groups.

Effect of Ethanol Intake on Renal AQP2, AQP3 and AQP4 mRNA and Diencephalon AVP mRNA Expression

Renal AQP2, AQP3 and AQP4 mRNA expression were examined by Northern analysis. As shown in Fig. 1, for AQP-2, -3 and -4, specific bands of expected size (1.5 Kb, 1.8 Kb and 5.5 Kb, respectively) (Fushimi et al., 1993) were detected. In the offspring, ethanol treatment increased the intensity of the bands corresponding to AQP2 and AQP3 mRNA by a factor of approx. 1.5.

In the ethanol-untreated progenitors, AQP2 mRNA levels were slightly lower than those in their offspring. Following prolonged ethanol ingestion, AQP2 and AQP3 mRNA levels increased by a factor of approx. 2 and 1.8, respectively (Fig. 1).

Renal AQP4 mRNA levels were not affected by ethanol treatment in any of the experimental groups.

AVP mRNA expression was also examined in the diencephalon of the offspring by Northern blot analysis and a specific single band of expected size (0.85 Kb) (Lefebvre & Zingg, 1991) was detected (Fig. 2). The intensity of this band was not affected by ethanol exposure during gestation and suckling periods.

The levels of cyclophilin mRNA, which were used to normalize AQP2 and AVP mRNA load in a particular lane, were not affected by ethanol.

Effect of Ethanol on Renal AQP2 Protein Expression

The Western blots of epithelial cell homogenates and apical membranes, obtained from the kidney of ethanol-treated and -untreated animals, are shown in Fig. 3. The anti-AQP2 antibody recognizes in the kidney the 29-, 35- and 50-kDa bands, which correspond to non-glycosylated and glycosylated AQP2 (Sasaki et al., 1994). Apical membrane AQP2 protein abundance was significantly increased (3-fold increase) in the offspring exposed to ethanol prenatally and during the suckling period, as compared with rats born from control animals (*see* Figs. 3 and 4). Ethanol exposure also increased slightly AQP2 protein levels in renal homogenates (*see* Figs. 3 and 4).

In the progenitors, ethanol increases AQP2 abundance in apical membranes by a factor of approx. 1.5 and slightly in the homogenates.

Discussion

Prolonged ethanol consumption alters fluid homeostasis and induces body dehydration (Wright & Donlon, 1979; Dow-Edwards et al., 1989; Magner et al., 1991; Carney et al., 1995); however, the molecular mechanism(s) involved in such derangement

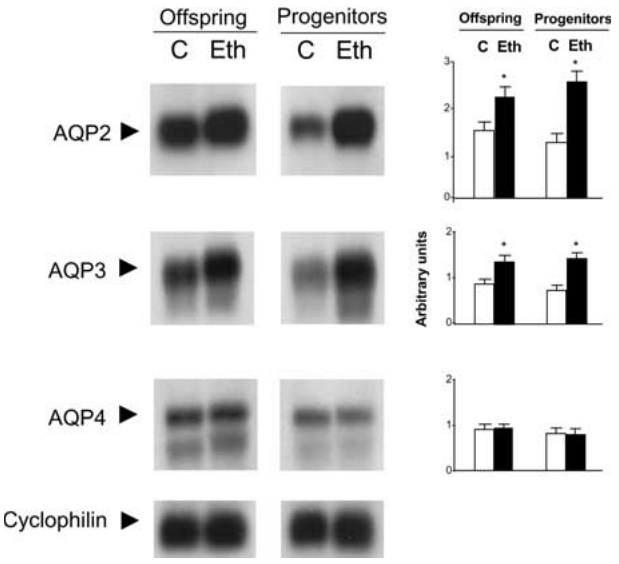

Fig. 1. A representative Northern blot of AQP2, AQP3 and AQP4 mRNAs in kidneys isolated from ethanol-treated (Eth) and untreated (C) progenitors and their respective offspring. 10 μg per lane of poly(A)$^+$ RNA were loaded onto the gel. The size of the transcripts was determined by ribosomal RNA. The AQP2, AQP3 and AQP4 transcripts displayed a motility corresponding to a size of ca. 1.5 Kb, 1.8 Kb and 5.5 Kb, respectively. *Bottom* laxes: The same blot was rehybridized with a riboprobe of cyclophilin. Histograms represent arbitrary values of mRNA levels after cyclophilin normalization. Means ± SEM, $n = 3$. *$p < 0.001$.

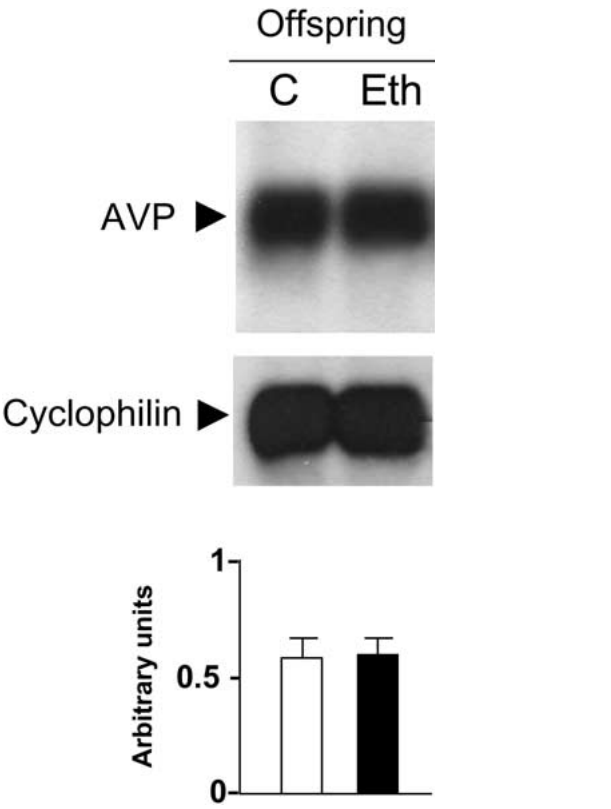

Fig. 2. A representative Northern blot of AVP mRNA in the diencephalon isolated from ethanol-treated (*Eth*) and untreated (*C*) offspring. 10 μg per lane of poly(A)$^+$ RNA were loaded onto the gel. The size of the transcripts was determined by ribosomal RNA. The AVP transcript displayed a motility corresponding to a size of ca. 0.85 Kb. Other details as in Figure 1. Means ± SEM, $n = 3$.

has(ve) not been investigated. The current study uses both, suckling rats born to mothers given ethanol before and during gestation and suckling periods and ethanol-treated progenitors, and the observations are compared to ethanol-untreated offspring and progenitors, respectively.

As reported before (Tavares et al., 1999), maternal ethanol consumption during gestation and suckling periods significantly decreased the offspring growth. The kidney weight was also significantly reduced. In the progenitors, the alcohol-induced changes in body and kidney weights were of lesser magnitude than in the offspring.

Ethanol treatment significantly reduced urine output and increased urine osmolality in both, suckling rats and progenitors. These ethanol-induced changes in urine parameters were greater in the suckling rats than in the progenitors.

Urine output and osmolality are mainly determined by proximal water reabsorption, corticopapillar osmotic gradient and collecting-duct water permeability. This permeability is mediated by the apical membrane AQP2 and the basolateral membrane AQP3 and AQP4. The current results reveal that ethanol treatment significantly increased renal papillary osmolality, AQP2 mRNA levels and AQP2 protein abundance in both, homogenate and apical membrane of renal epithelial cells. Ethanol treatment also increased renal AQP3 mRNA levels in both, progenitors and offspring, whereas those of AQP4 were not affected. Therefore, ethanol treatment affected AQP2 and AQP3 at the gene level (increased their mRNA levels) and stimulated the targeting of AQP2 to the apical membrane of the collecting duct cells.

AVP is the main regulator of AQP2 at the gene, protein and cell level (*see* Ward et al., 1999; Nielsen et al., 2002 for reviews). Conditions that increase plasma osmolality, such as dehydration, increased plasma levels of AVP (Rehbein et al., 1986). As ethanol-treated progenitors drink significantly less water than control animals (the amount of milk ingested by the offspring was not determined), it could be thought that the observed renal AQP2 upregulation would result from increased AVP circulating levels produced in response to ethanol-induced body dehydration. However, neither plasma osmolality, nor plasma levels of AVP nor AVP mRNA expression were significantly affected in ethanol-exposed offspring as compared with control offspring. These observations agree with previous reports (Ruela et al., 1994; Silva et al., 2002) showing that though numerous neurons

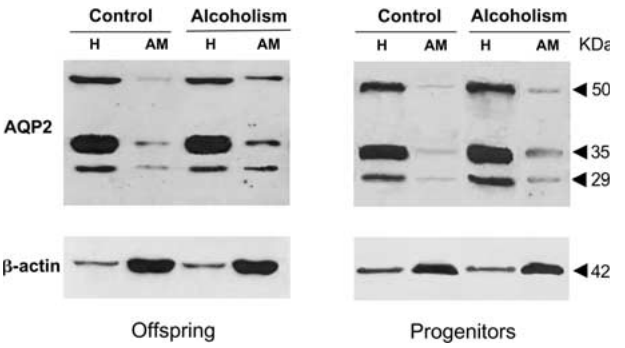

Fig. 3. A representative Western blot of homogenates (*H*) and apical membranes (*AM*) isolated from kidneys of both, control and ethanol-treated progenitors and their offspring. A total of 70 μg of protein was loaded onto the gel per lane. The blots were probed with the polyclonal anti-COOH-terminal AQP2 antibody and with the anti-β-actin antibody, as described in the Methods section.

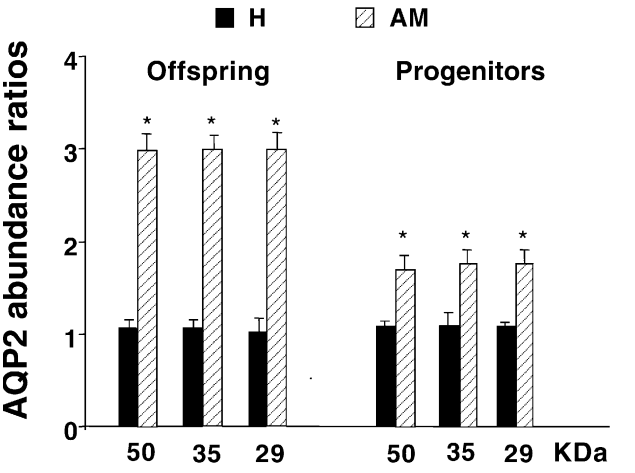

Fig. 4. Ethanol *vs.* control ratios of AQP2 protein abundance in renal homogenates (*H*) and apical membranes (*AM*). AQP2 relative abundance in homogenates (*H*) and apical membranes (*AM*) were normalized by β-actin. The ratios were calculated by dividing the values of normalized signal intensity of the two conditions that are compared. Means ± SEM, $n = 3$. *$p < 0.001$ apical membranes *vs.* homogenates.

of the hypothalamic magnocellular system degenerate after prolonged ethanol exposure, the AVP mRNA levels were not affected, due to compensatory up-regulation of AVP gene expression undergone by the surviving neurons.

So far, the current results suggest that the ethanol-induced AQP2 upregulation and increase in renal papillary osmolality are not mediated by changes in AVP plasma levels. Increased levels of AQP2 expression despite normal levels of circulating AVP have been reported in pregnant rats (Ohara et al., 1998). Neither does aldosterone appear to play a significant role in these changes, because it was only significantly increased ($p < 0.05$) in ethanol-exposed offspring, but not in the ethanol-treated progenitors. The corticopapillary osmotic gradient has been considered to contribute to increased AQP2 expression (Uchida et al., 1994). Also, ethanol has been shown to induce cAMP-dependent gene expression (Dohrman, Diamond & Gorman, 1996; Asher et al., 2002) and it is well established that cAMP regulates movement of AQP2-containing vesicles to the plasma membrane (*see* Ward et al., 1999; Nielsen et al., 2002 for reviews). Alternatively, other intra-renal ethanol effects may account for the ethanol-induced anti-diuresis. Thus, ethanol may modify either the physical state of membrane lipids or the cell membrane fluidity (Goldstein, 1984), which in turn may affect V2 receptor activity or cell protein trafficking.

It is well documented in human as well as in experimental animals that the urine-concentrating ability decreases with aging. This decrease has been shown to be associated with a down-regulation of AQP2 expression (Terashima et al., 1998), which in turn is independent of AVP-mediated cAMP accumulation (Preisser et al., 2000). In agreement with these previous observations, the present study reveals that the 21-day-old rats present higher ability to concentrate urine and also increased response of AQP2 expression to ethanol than 5-month-old rats.

We concluded that maternal ethanol ingestion before and during gestation and suckling periods affects the renal function of the offspring. At the molecular level ethanol treatment appears to upregulate renal AQP2 and AQP3 expression by an AVP-independent mechanism. Progenitors manifest similar responses to ethanol although of lesser magnitude than the offspring. These observations may contribute to the understanding of the molecular basis underlying the ethanol-induced changes in water-electrolyte homeostasis.

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