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The effect of water deprivation on the tonicity responsive enhancer binding protein (TonEBP) and TonEBP-regulated genes in the kidney of the Spinifex hopping mouse, *Notomys alexis*

Ray C. Bartolo* and John A. Donald

School of Life and Environmental Sciences, Deakin University, Geelong, Victoria 3217, Australia

*Author for correspondence at present address: Department of Physiology, University of Otago, PO Box 913, Dunedin, 9054, New Zealand (e-mail: ray.bartolo@otago.ac.nz)

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SUMMARY

In desert rodents, the production of concentrated urine is essential for survival in xeric environments in order to conserve water. Reabsorption of water in the kidney is dependent on large osmotic gradients in the renal medulla. This causes the renal cells to be bathed in a hypertonic extracellular fluid that can compromise cellular function. In response to hypertonicity, kidney cells accumulate compatible, non-ionic osmolytes that lower the ionic strength within the cells to isotonic levels by replacing intracellular ionic electrolytes. The tonicity-responsive enhancer binding protein (TonEBP) is a transcription factor that regulates the expression of genes that encode proteins that catalyse the accumulation of compatible osmolytes. We investigated the expression of TonEBP mRNA and protein and compatible osmolyte genes in the Spinifex hopping mouse, *Notomys alexis*, an Australian desert rodent that produces a highly concentrated urine. TonEBP mRNA expression was unchanged after 3 days of water deprivation but was significantly increased after 7 and 14 days of water deprivation. Immunohistochemistry showed that during water deprivation TonEBP had translocated from the cytoplasm into the nucleus of cells in the renal medulla and papilla. In addition, 3, 7 and 14 days of water deprivation caused a significant increase in aldose reductase (AR), *myo*-inositol (SMIT), betaine/GABA (BGT-1) and taurine (TauT) transporter mRNA expression, which is indicative of an increase in TonEBP activity. In desert rodents, TonEBP regulation of gene transcription is probably an important mechanism to protect renal cells in the face of the large corticomedullary gradient that is required to concentrate urine and conserve water.

Key words: *Notomys alexis*, tonicity responsive enhancer binding protein (TonEBP), compatible osmolytes, kidney, water deprivation.

INTRODUCTION

Desert mammals can survive for long periods without free water by obtaining preformed water from food and metabolic water from the oxidation of hydrogen, and by reducing the amount of water lost during respiration and excretion (Degen, 1997). The excretion of highly concentrated urine is dependent on the regulation of two processes: ultrafiltration of plasma in the glomerulus and reabsorption of water in the renal tubules. Accordingly, desert mammals have a reduced glomerular filtration rate and enhanced tubular water reabsorption (Degen, 1997). Water reabsorption relies on the medullary osmotic gradient created by the loop of Henle (Bankir and Rouffignac, 1985). Desert rodents, in particular, have long loops of Henle, and routinely excrete urine in the range of 4000–7000 mOsm l^{-1} (Degen, 1997). A high osmolality of the inner medulla and papilla of the kidney exposes the cells in that region to a hypertonic environment, which can severely compromise cell function and lead to DNA damage and apoptosis (Burg et al., 2007; Jeon et al., 2006). The most abundant osmolytes in the renal medulla and papilla of the kidney are Na^+, Cl^- and urea (Garcia-Perez and Burg, 1991). High NaCl causes hypertonicity and disturbances in cell volume, but urea has no effect on tonicity as it readily permeates cell membranes (Yancey et al., 1982).

In response to hypertonicity, renal cells will rapidly accumulate inorganic ions, but in conjunction with elevated urea concentrations, this will compromise the function of cellular proteins and macromolecules (Yancey et al., 1982). The intracellular ionic concentration is then lowered by the replacement of the inorganic ions with compatible osmolytes, which occurs over 1–3 days (Rauchman et al., 1997; Jeon et al., 2006; Burg et al., 2007). Since compatible osmolytes do not contribute to ionic strength, the intracellular ionic concentration remains within an optimal physiological range, while the osmolality of the interstitium and intracellular fluid is the same (Beck et al., 1998; Garcia-Perez and Burg, 1991). The accumulation of compatible osmolytes is reversible since rehydration leading to diuresis, causes swelling of the medullary cells and the release of compatible osmolytes into the interstitial fluid (Beck et al., 1998; Garcia-Perez and Burg, 1991).

Sorbitol (D-glucitol), *myo*-inositol, betaine and taurine are compatible osmolytes that are found in abundance in the renal medulla. In response to hypertonicity, sorbitol is produced from glucose by the aldose reductase (AR) enzyme, whereas *myo*-inositol, betaine and taurine are transported into cells by Na^+- or Na^+/Cl^--dependent transporters (Burg et al., 1996). The transcription of AR and the *myo*-inositol (SMIT; also known as SLC5A3), betaine/GABA (BGT-1; also known as SLC6A12) and taurine (TauT; also known as SLC6A6) transporters is regulated by the tonicity-responsive enhancer binding protein (TonEBP; also known as NFAT5) (Burg et al., 1996). The AR, SMIT, BGT-1 and TauT genes have osmotic response elements (ORE) in their 5′ flanking regions that contain the tonicity-responsive enhancer (TonE) consensus sequence (Takenaka et al., 1994). The binding of TonEBP to OREs increases the transcription of AR, SMIT, BGT-1 and TauT,

which in turn leads to an increase in the intracellular accumulation of the respective compatible osmolyte (Woo and Kwon, 2002; Woo et al., 2002). TonEBP has a basal activity level under isotonic conditions that is decreased by hypotonicity and increased by hypertonicity. An increase in TonEBP activity is reflected by an increase in the expression of AR, SMIT, BGT-1 and TauT mRNAs; mice lacking the TonEBP gene have a reduced expression of AR, SMIT, BGT-1 and TauT (Lopez-Rodriguez et al., 2004). The bidirectionality of TonEBP activity has been demonstrated in Madin-Darby canine kidney (MDCK) cells (Woo et al., 2000a). In MDCK cells grown under isotonic conditions, TonEBP is distributed between the nucleus and cytoplasm, but under hypertonic stress TonEBP increases in abundance and translocates to the nucleus to act as a transcription factor of osmoprotective genes. By contrast, when the MDCK cells are transferred to a hypotonic medium, TonEBP translocates to the cytoplasm, its abundance decreases, and compatible osmolytes move out of the cell (Woo et al., 2000a).

The Spinifex hopping mouse, *Notomys alexis*, is a small rodent that is highly adapted to survive in arid environments where it can live without drinking water (MacMillen and Lee, 1969; Weaver et al., 1994). *N. alexis* has been reported to produce the most concentrated urine of any mammal (9370 mOsm l^{-1}) (MacMillen and Lee, 1969). In the laboratory, *N. alexis* can tolerate water deprivation for 28 days without changes in plasma osmolality, vasopressin or renin, as seen in other species of desert rodent (Weaver et al., 1994; Heimeier et al., 2002). By contrast, water deprivation experiments using laboratory rats and mice are short-term because of the inability of the animals to survive without drinking, and the animals show a marked increase in plasma osmolality and vasopressin levels (see Degen, 1997). Thus, the mechanisms underpinning survival during long-term water deprivation in desert mammals may be different from mesic species. Given the extraordinary urine-concentrating ability of *N. alexis*, we predicted that the expression of TonEBP and the genes encoding proteins that regulate intracellular compatible osmolytes would play an important role and be upregulated in the renal medulla of *N. alexis* during periods of water stress. Thus, the aim of the current study was to analyse the expression of TonEBP, AR, BGT-1 SMIT and TauT mRNAs and determine the distribution of TonEBP protein in the kidney of water-deprived *N. alexis*, in comparison to mice with *ad libitum* access to water.

MATERIALS AND METHODS

Animals

Spinifex hopping mice *Notomys alexis* Thomas 1922 were obtained from a breeding colony at the Deakin University Animal House, Geelong, Australia. The animals were housed in rat boxes containing straw and sawdust for bedding with wire mesh lids at 21–24°C and a 13 h:11 h light:dark cycle (lights on at 08:00 h). The animals received fresh tapwater *ad libitum* and were fed French White millet seed.

Water deprivation experiments

All experiments involving *N. alexis* were performed with approval from the Deakin University Animal Welfare Committee (Project number A31/2003). The water deprivation experiments were carried out as previously described (Donald and Bartolo, 2003). For each water deprivation time point, there was a water-replete (control) group in which *N. alexis* had *ad libitum* access to water, and a water-deprived (experimental) group in which *N. alexis* was subjected to 3, 7 or 14 days without access to free water; this was termed water deprivation. All mice were ear-tagged to enable identification, and

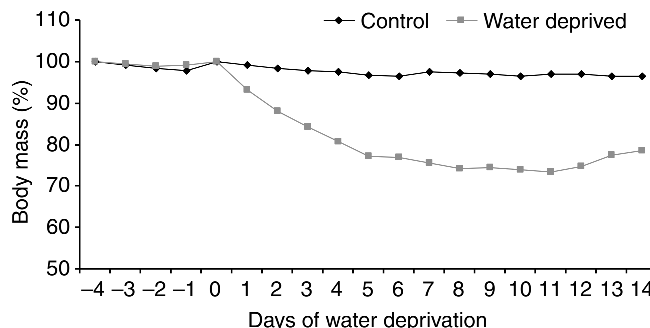

Fig. 1. The effect of 14 days of water deprivation on body mass in *N. alexis*. There was a significant decrease in mean body mass during the initial period of water deprivation, but after day 7 it stabilised and began to increase. Water-replete *N. alexis* showed no significant change in body mass.

were approximately 6 months old. There were eight animals in each group and they were housed in groups of four in sand-filled glass aquaria (W 100 cm×H 40 cm×L 50 cm), which allowed for communal sleeping burrows. The mice were weighed and fed 20 g of millet seed per cage daily; a group of four mice eat a maximum of 4.5 g seed per day during water deprivation (R.C.B., unpublished). The mass of the animals in the 14-day water deprivation experiment was used for the analysis shown in Fig. 1. At the end of the respective water deprivation periods, the mice were anaesthetised by halothane inhalation followed by cervical dislocation. The kidneys were dissected free, and the left kidney was frozen in liquid nitrogen and stored at –80°C until RNA was isolated, while the right kidney was fixed overnight at 4°C in 4% formaldehyde (pH 7.4; ~1100 mOsm kg^{-1}), and then stored in 70% ethanol until processing for immunohistochemistry.

Collection of urine for the measurement of osmolality

A separate water deprivation experiment using six *N. alexis* was performed to measure urine osmolality. The mice were water-deprived for 14 days and urine was collected on day 0, 3, 7 and 14. Urine was collected from individual mice placed for 1 h in cylindrical containers (90 mm diameter and 150 mm high) that had a wire mesh floor. The containers were suspended above a collection tray that contained light paraffin oil, thus preventing evaporation of the urine. Urine osmolality was then measured using a Vapro® vapour pressure osmometer (Wescor Model 5520, Logan, UT, USA).

Amplification, cloning and sequencing of putative cDNAs

Kidney total RNA was isolated using TRIzol (Invitrogen, Mount Waverley, Victoria, Australia), which utilises the single step acid guanidinium thiocyanate-phenol-chloroform extraction method (Chomczynski and Sacchi, 1987). The RNA concentration was determined by spectrophotometry at 260 nm. First strand cDNA was synthesised from kidney total RNA using Superscript II (Invitrogen) as per the manufacturer's protocol. Primers were designed based on *Mus musculus* sequence data obtained from GenBank (National Centre for Biotechnology, NCBI). The accession numbers for the *M. musculus* sequences are as follows: TonEBP, AF453571; AR, NM_009658; BGT-1, NM_133661; SMIT, AF220915; and TauT, AAB54039.

Primer sequences, annealing temperature, and the size of the predicted PCR amplicons are shown in Table 1. PCR was performed in a total volume of 20 μl with a final concentration of: 1× PCR

Table 1. PCR amplification of TonEBP, AR, BGT-1, SMIT and TauT from *N. alexis*

	Forward primer (5′–3′)	Reverse primer (5′–3′)	AT	PS (bp)
TonEBP	ATGCAATTTCAGAATCAGCC	GCATTTGCTGAGAAAGAAG	60°C	514
AR	TTGACTGCGCCCAGGTGTAC	TATATGCTGTCACCACGATGC	60°C	504
BGT-1	ATGGACCAGAAAGGCAAGGAC	CTCTCCCAGAATTCCATGACAG	60°C	509
SMIT	CACTGTGAGTGGATACTTCC	TCTCTTAACTTCCTCAAACC	52°C	544
TauT	TCCACAAAGACATCCTGAAGC	GGTGAAGTTGGCAGTGCTAAG	60°C	539

AR, aldose reductase; BGT-1, betaine/GABA transporter; SMIT, *myo*-inositol transporter; TauT, taurine transporter; TonEBP, tonicity-responsive enhancer binding protein; AT, annealing temperature; PS, expected size of the PCR amplicons (bp).

buffer, 0.2 mmol l^{-1} dNTPs, 1 μmol l^{-1} of each forward and reverse primer, 1.0 i.u. of Taq DNA polymerase (Scientifix, Melbourne, Australia), 2.5 mmol l^{-1} $MgCl_2$ and 1 μl of the cDNA synthesis reaction. Amplification of the various cDNAs was performed as follows: initial denaturation of 300 s at 94°C, 35 cycles of 45 s at 94°C, 30 s at the annealing temperature (Table 1), 45 s at 72°C, and a final extension of 300 s at 72°C. The PCR products were purified and cloned into a pCR2.1 vector, which was then transformed into One Shot TOP10 chemically competent *Escherichia coli* cells using a TA cloning® Kit (Invitrogen). The cloned cDNAs were sequenced on an Applied Biosystems automated sequencer (Australian Genome Research Facility, Brisbane, Australia). The BLAST (Basic Local Alignment Search Tool) program on the NCBI database was used to search GenBank for similar sequences (Altschul et al., 1997). Alignments of *N. alexis* and *M. musculus* nucleotide and amino acid sequences were carried out to determine homology between cloned *N. alexis* cDNAs and the sequences from which PCR primers were designed, using ClustalW (http://www.ebi.ac.uk/clustalw/).

mRNA expression analysis

Reverse transcription PCR was used to detect changes in the expression of TonEBP, AR, BGT-1, SMIT and TauT mRNAs in the kidney of water-replete and water-deprived *N. alexis*. Total RNA isolation and cDNA synthesis were performed as described above. For the analysis of mRNA expression, 2 μg of total RNA was reverse transcribed, and then 1 μl of the cDNA reaction was used in PCR. To quantify the level of mRNA expression between control and water-deprived *N. alexis*, the expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA was used as an internal control, as the expression of *GAPDH* does not change in response to water deprivation (Heimeier et al., 2002; Sturzenbaum and Kille, 2001). The *GAPDH* primers were as follows: forward 5′-GAAGGTCGGTGTGAACGGATTTG-3′, and reverse 5′-TTACTCCTTGGAGGCCATGTAGG-3′; these primers generated a 999 base pair amplicon.

In preliminary experiments, the linear amplification range of the genes of interest and GAPDH were determined by running PCR reactions for a varying number of cycles between 17 and 35. The linear range was also determined in samples from control and water-deprived mice to ensure that there was no variation between individual animals or sample groups. The PCRs for gene expression analysis were performed for varying numbers of cycles, depending on the gene: GAPDH, 20; TonEBP, 22; AR, 24; BGT-1, 28; SMIT, 24; TauT, 31. The parameters for the PCR cycles were the same as previously mentioned.

For the quantification of the PCR products, the reactions were spiked with 2.5 μCi of [α-^{32}P]dCTP. As the radio-labelled dCTP is randomly incorporated into the PCR products, the level of radiation emitted by a PCR product is directly proportional to the amount of PCR product. Equal aliquots of the GAPDH PCR and gene of interest PCR were mixed (to avoid gel loading error) and subjected to electrophoresis on a 1.5% agarose gel with a 1× Tris–borate–EDTA (TBE) running buffer at 100 V. The gel was incubated in 0.5 μg ml^{-1} of ethidium bromide, visualised on an UV light box and the cDNA bands were excised, and placed in microcentrifuge tubes. The amount of isotope [α-^{32}P] incorporated into the PCR products was measured by placing the microcentrifuge tubes in vials and counting in a scintillation counter (Tri-Carb 2000CA Liquid Scintillation Counter, United Technology Packard, Downers Grove, IL, USA). The expression of the various mRNAs was determined as a ratio of GAPDH mRNA expression (gene of interest/GAPDH), and the difference in the ratios between water-replete and water-deprived groups were analysed for statistical significance. The mRNA expression data are expressed as a percentage of the control where the mean values from control animals represent 100% for illustrative purposes only.

TonEBP Immunohistochemistry

One kidney from all mice (N=8 for each time point) was analysed for TonEBP immunoreactivity (TonEBP-IR). Fixed tissues were processed in a Leica TP 1010 automated tissue processor (Wetzlar, Germany), which dehydrated the tissue through a series of ethanol and xylene washes. The kidneys were then embedded in Paraplast™ tissue embedding medium, and 5 μm sections were placed on slides coated in 2% 3-aminopropyltiethoxysilane (Sigma) and allowed to dry overnight. Sections were prepared for immunohistochemistry by dewaxing in xylene and rehydration through a graded series of ethanol to water. Endogenous peroxidase activity was quenched by incubating the sections in 3% hydrogen peroxide for 10 min. The sections then underwent heat-induced epitope retrieval; sections were incubated in 1.0 mmol l^{-1} EDTA buffer (pH 8.0) for 10 min, heated for 3×5 min in a 650 W microwave oven, cooled to room temperature and washed in phosphate-buffered saline (PBS; pH 7.4; 2×5 min washes). Endogenous biotinylated proteins were blocked by the use of an Avidin–Biotin blocking kit (Vector Laboratories, Burlingame, CA, USA), which involved incubating sections in Avidin D solution for 15 min, a 1 min rinse with PBS, and incubation in Biotin solution for 15 min, followed by incubation with an affinity-purified rabbit anti-mouse TonEBP antiserum for 2 h at room temperature. The TonEBP antiserum was diluted (1:5000) with PBS, and was kindly donated by Prof. Seung Kyoon Woo, University of Maryland, Baltimore, USA (Miyakawa et al., 1999). The sections were then washed in PBS for 2×10 min. A Vectastain ABC kit (Vector Laboratories) was used for the detection of the TonEBP antiserum. The sections were incubated with biotinylated secondary antibody solution (1:200) for 30 min, washed in PBS for 2×10 min, and incubated with the Vectastain ABC reagent (Vector Laboratories) for 45 min. Sections were then washed in PBS for 10 min, rinsed in 0.1 mol l^{-1} Tris (pH 7.4) and incubated in 0.02% diaminobenzidine tetrahydrochloride (DAB; in 0.1 mol l^{-1} Tris, pH 7.4) for 10 min. The slides were examined under a light microscope (Axioskop 20, Carl Zeiss, Göttingen, Germany)

and sections were photographed with a digital colour system (Spot 35 Camera System, Diagnostic Instruments, Sterling Heights, MI, USA). The specificity of staining was determined by running negative controls omitting primary and/or secondary antibody.

Data analysis

To test the difference in mRNA expression between control and experimental groups, a Student's *t*-test was performed. Changes in body mass during the 14-day water deprivation experiment were analysed using a two-way ANOVA and a Student's *t*-test, and urine osmolalities were analysed using a one-way ANOVA; each used a Tukey's *post-hoc* test. All statistical probabilities were calculated using SPSS for Windows 14, and $P\leqslant 0.05$ was considered significant (Quinn and Keogh, 2002).

Materials

[α-^{32}P]dCTP (3000 Ci mmol l^{-1}) was purchased from GE Life Sciences (Rydalmere, NSW, Australia). The Vectastain ABC kit was purchased from Abacus ALS, Brisbane, Australia. All other chemicals were either reagent or molecular grade and were purchased from Sigma-Aldrich (Castle Hill, NSW, Australia) or Scientifix (Melbourne, Australia).

RESULTS

Cloning and sequencing of *N. alexis* cDNAs

TonEBP, AR, BGT-1, SMIT and TauT cDNAs were cloned and sequenced from *N. alexis* kidney cDNA. The nucleotide and deduced amino acid sequences of the cDNAs are available form the NCBI GenBank; accession numbers for the respective sequences are shown in Table 2. Sequence analysis of the cloned *N. alexis* cDNAs showed that they have high homology to the respective *M. musculus* sequences from which the PCR primers were designed. Table 2 also summarises the sequence homology (as a percentage) of *N. alexis* nucleotide and deduced amino acid sequences, to their respective *M. musculus* sequences.

Table 2. NCBI GenBank accession numbers for partial *N. alexis* TonEBP, AR, BGT-1, SMIT and TauT nucleotide and deduced amino acid sequences, and homology to *Mus musculus*

	Nucleotide		Amino acid	
	Accession no.	% Similarity to *Mus*	Accession no.	% Similarity to *Mus*
TonEBP	AY856060	97.3	AAW47642	95.6
AR	AY856068	96.1	AAW47650	97.4
BGT-1	AY856066	94.4	AAW47648	97.4
SMIT	AY856065	96.4	AAW47647	98.8
TauT	AY856067	99.6	AAW47649	99.4

AR, aldose reductase; BGT-1, betaine/GABA transporter; SMIT, *myo*-inositol transporter; TauT, taurine transporter; TonEBP, tonicity-responsive enhancer binding protein.

Effect of water deprivation on body mass and urine osmolality of *N. alexis*

Control (water-replete) *N. alexis* showed no significant change in body mass over the course of the experiment (Fig. 1). By contrast, *N. alexis* subjected to water deprivation lost mass over the first 7 days, after which it stabilised (Fig. 1, Table 3). Compared to the body mass at the beginning of water deprivation, there was a significant decrease in mass after 3, 7 and 14 days of water deprivation; body mass at day 7 and day 14 was significantly less than that at day 0 and day 3, respectively. Mean urine osmolality significantly increased in response to 3, 7 and 14 days of water deprivation, when compared to water-replete *N. alexis* (Table 3). The urine osmolality at day 14 was significantly higher than that at day 0, and 3 and 7 days of water deprivation (Table 3).

Table 3. Percentage change in body mass and urine osmolality in *N. alexis* after 3, 7 and 14 days of water deprivation

	% Body mass decrease	Urine osmolality (mOsm kg^{-1})
Day 0	–	979±131
3-day WD	15.6±3.5*	3532±212‡
7-day WD	24.6±3.7†	4109±190‡
14-day WD	21.3±8.1†	5346±336§

WD, water deprivation; *a significant difference from day 0; †a significant difference from 0 and 3 days of water deprivation: ‡a significant difference from day 0; §a significant difference from 0, 3 and 7 days of WD.

TonEBP mRNA expression and protein immunolocalisation in the kidney

TonEBP mRNA expression was unaffected (P=0.806) by 3 days of water deprivation, but there was a significant increase in its expression after 7 (P=0.013) and 14 (P<0.001) days of water deprivation (Fig. 2).

In the renal cortex of water-replete mice, weak TonEBP-IR was observed in the tubular epithelial cells, and the nuclear and cytoplasmic distribution appeared the same (Fig. 3A). The distribution and intensity of TonEBP-IR in the renal cortex of *N. alexis* deprived of water for 3, 7 and 14 days was similar to that of water-replete mice (Fig. 3B). In the outer medulla of water-replete *N. alexis*, TonEBP-IR was more distinct in the nuclei than cytoplasm of the collecting duct epithelial cells (Fig. 3C). After 3, 7 and 14 days of water deprivation, there was a marked increase in TonEBP-IR in the nuclei of the collecting duct epithelial cells, compared to that

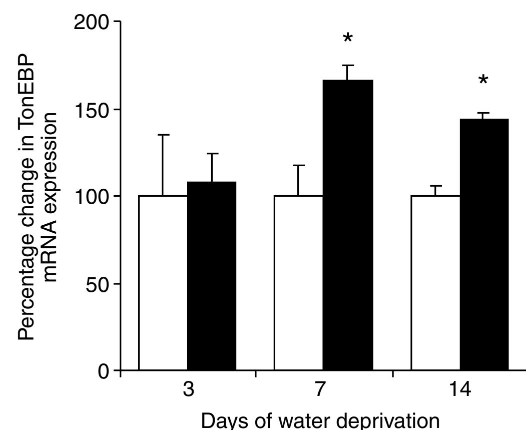

Fig. 2. Histograms showing the relative expression of TonEBP mRNA in control (open bars) and water-deprived (closed bars) *N. alexis* kidney. The ratio of TonEBP to GAPDH for control *N. alexis* was set to equal 100%, and the ratio of the water-deprived *N. alexis* is represented as a percentage of control values. *Statistically significant change in TonEBP mRNA expression from control values ($P\leqslant 0.05$). A significant increase in TonEBP mRNA expression was shown in the kidney after 7 and 14 days of water deprivation, but no change was detected after 3 days of water deprivation.

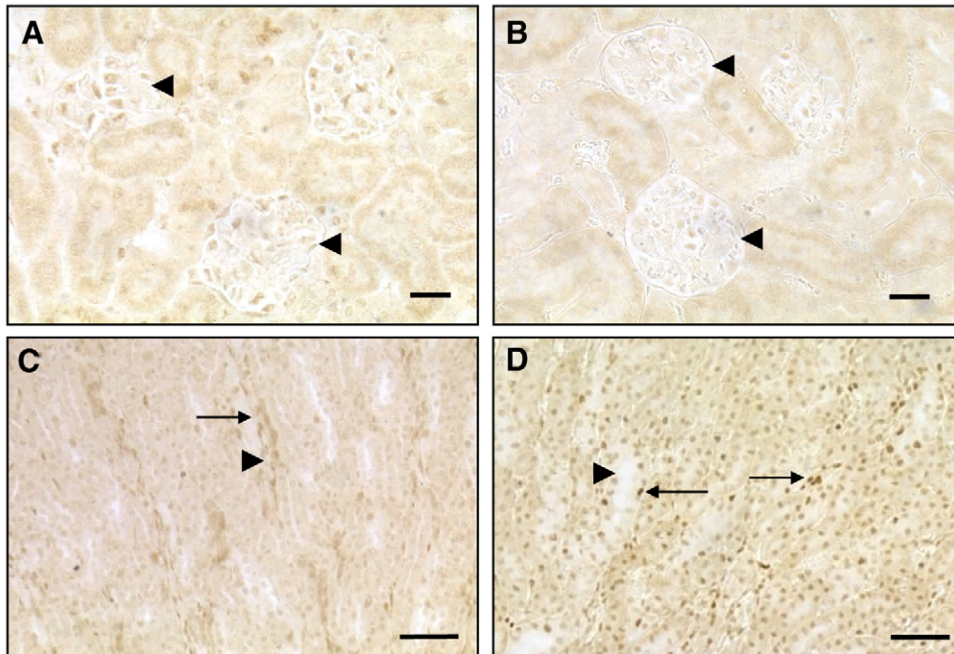

Fig. 3. Light micrographs of 5 μm thick sections showing TonEBP immunoreactivity (TonEBP-IR) in the renal cortex (A,B) and outer medulla (C,D) of control (A,C) and 14-day water-deprived *N. alexis* (B,D). In A and B, black arrowheads indicate the glomeruli. TonEBP-IR is weak in the renal cortex and is evenly distributed between the cytoplasm and nucleus of control (A) and 14-day water-deprived mice (B). In C and D, black arrows indicate the nuclei of epithelial cells lining the tubules that pass through the outer medulla, and arrowheads indicate the tubule lumen. Moderate TonEBP-IR can be observed in the nuclei and cytoplasm in the epithelial cells lining the renal tubules of control *N. alexis* (C), but TonEBP-IR was more intense in the nuclei than the cytoplasm of the epithelial cells in the outer medulla of 14-day water-deprived mice (D). Scale bars, 30 μm (A,B), 120 μm (C,D).

in water-replete animals; the nuclei of 14-day water-deprived mice showed the most intense TonEBP-IR staining (Fig. 3D). Similar patterns of TonEBP-IR staining were observed in the inner medulla (Fig. 4A–D). Control experiments confirmed that TonEBP immunolocalisation was specific.

Effect of water deprivation on transcription of AR, BGT-1, SMIT and TauT mRNAs

The expression of AR, BGT-1, SMIT and TauT mRNAs was analysed in the kidney of water-replete *N. alexis*, and compared to that in 3-, 7- and 14-day water-deprived mice. The expression of each gene was significantly increased ($P<0.05$) after each period of water deprivation (Fig. 5).

DISCUSSION

The high osmolalities in the renal medulla that are essential for the production of a concentrated urine create a hostile environment for renal cells because of the deleterious effects of high ionic concentrations on cellular function. TonEBP regulates the transcription of genes that facilitate the intracellular accumulation of compatible osmolytes, which counteract the effects of high concentrations of non-compatible osmolytes such as sodium (Burg

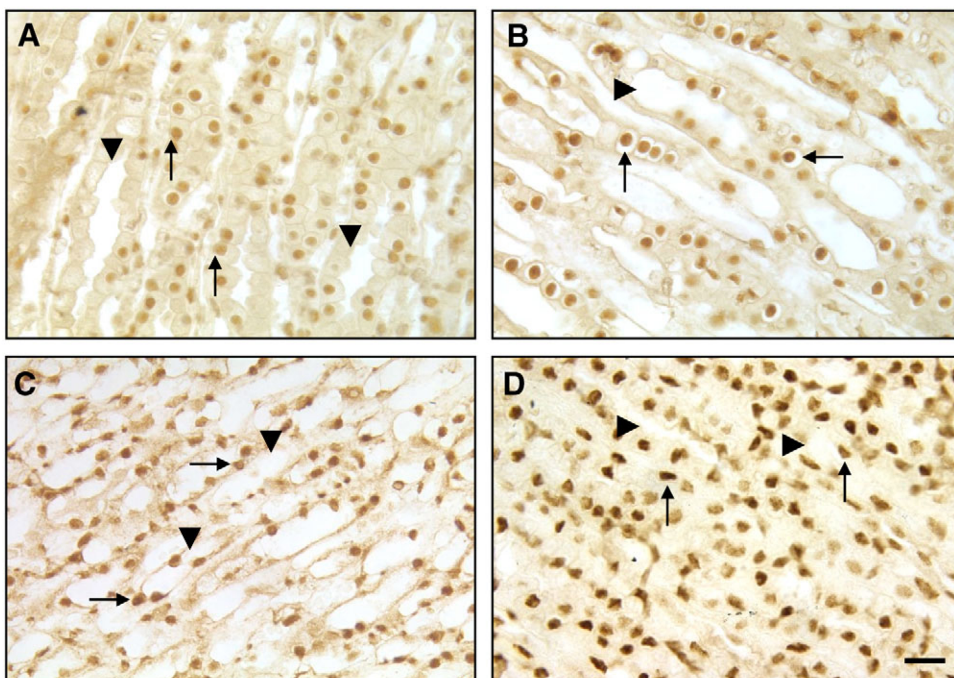

Fig. 4. Light micrographs of 5 μm thick sections illustrating TonEBP immunoreactivity (TonEBP-IR) in the inner medulla of control (A) and 3- (B), 7- (C) and 14- (D) day water-deprived *N. alexis*. Black arrows indicate the nuclei of the collecting duct epithelial cells that pass through the renal papilla (A–D), and arrowheads indicate the lumen of the collecting ducts. TonEBP-IR was quite strong in the nuclei of the collecting duct epithelial cells in the inner medulla of control mice (A). The intensity of TonEBP-IR in the nuclei increased as the water deprivation period was prolonged (B–D). Scale bar, 30 μm.

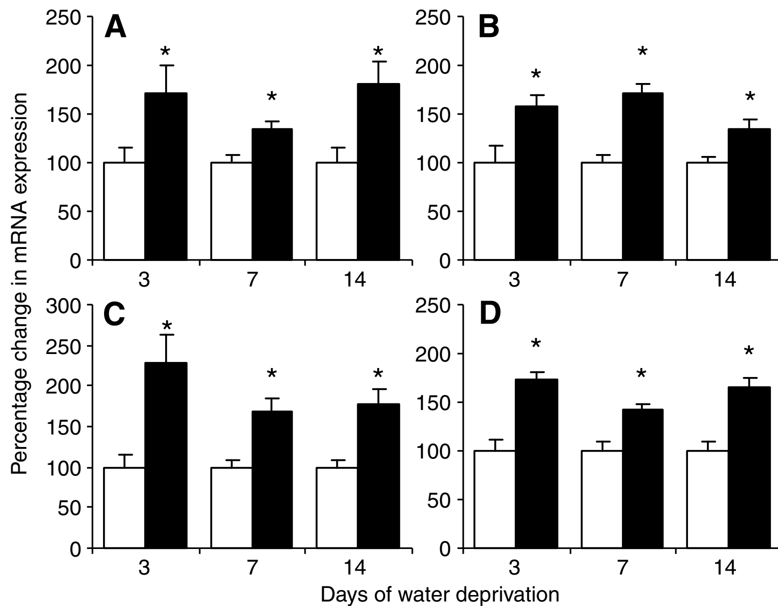

Fig. 5. Histograms showing the relative expression of AR (A), BGT-1(B), SMIT (C) and TauT (D) mRNA in control (open bars) and water-deprived (closed bars) *N. alexis* kidney. The ratios of the mRNA of interest to GAPDH mRNA for control *N. alexis* were set to equal 100%, and the ratios for the water-deprived *N. alexis* are represented as a percentage of control values. *Statistically significant change in the expression of gene of interest from control values ($P \leqslant 0.05$). A significant increase in the expression of AR, BGT-1, SMIT and Taut mRNA was found in the kidney of 3-, 7- and 14-day water-deprived *N. alexis*.

et al., 2007; Jeon et al., 2006). We found that water deprivation in *N. alexis* caused an upregulation of TonEBP mRNA expression, translocation of TonEBP to the nucleus of renal medullary cells, and an increased expression of AR, SMIT, BGT-1 and TauT mRNAs. The response would be an essential physiological mechanism that protects renal medullary cells and contributes to the ability of rodents such as *N. alexis* to tolerate prolonged periods of water deprivation.

In order to study the expression of TonEBP, AR, BGT-1, SMIT and TauT mRNAs in *N. alexis*, partial cDNAs encoding each gene were initially cloned and sequenced. All *N. alexis* cDNAs sequenced in this study showed greater than 94% nucleotide sequence homology to the *M. musculus* sequences from which the respective PCR primers were designed, and at least 95% amino acid sequence homology to the respective *M. musculus* protein sequences. Previous sequencing of hopping mouse cDNAs encoding regulatory hormones and receptors has shown high sequence identity to *M. musculus* (e.g. Donald and Bartolo, 2003; Heimeier et al., 2002); both species are Old World rodents and members of the family Muridae and the subfamily Murinae.

During water deprivation, *N. alexis* lost mass until day 7, at which point body mass stabilised and began increasing up to day 14, which is consistent with previous water deprivation studies with hopping mice (Heimeier and Donald, 2006). During water deprivation, body fat is metabolised to increase the production of metabolic water, which will contribute to the maintenance of water balance (Degen, 1997). In *N. alexis*, the loss of body fat in the early stages of water deprivation is the main component of the observed weight loss (R.C.B., unpublished). The ability of desert rodents to produce extremely concentrated urine during water deprivation is a critical factor that contributes to the conservation of body water. In the current study, *N. alexis* was able to significantly increase urine osmolality during water deprivation, reaching a maximum of 6348 mOsm kg^{-1} at 14 days. Although this is well below the previously reported maximal value for *N. alexis* urine, of 9370 mOsm kg^{-1}, MacMillen and Lee (MacMillen and Lee, 1969) reported a mean value of 6550±510 mOsm kg^{-1} for hopping mice water-deprived for 21 days. This is comparable to the mean of 5346±336 mOsm kg^{-1} from the 14-day water-deprived animals in this study. In desert rodents, urine concentration can be influenced by the length of the water deprivation period, the salt and protein content of the diet (Gamble et al., 1929; Gamble et al., 1934) and the housing arrangement of the mice. In particular, communal nesting of *N. alexis* has been shown to be an important behavioural adaptation that leads to a lowering of metabolic rate (up to 18%) and a reduction in pulmo-cutaneous water loss (up to 25%), compared to mice housed individually (Baudinette, 1972). In the current study, the mice were housed in groups of four, but MacMillen and Lee (MacMillen and Lee, 1969) housed the mice individually, which may partly explain the very high urine osmolalities recorded in their study.

The expression of TonEBP mRNA in the kidney of 3-day water-deprived *N. alexis* was not significantly different from that of *N. alexis* with access to water, which is consistent with the findings of a study performed with rats that also found no change in the expression of TonEBP mRNA after 3 days of water deprivation (Cha et al., 2001). The results of the current study and that of Cha et al. (Cha et al., 2001), would suggest that the stimulus for increasing the transcription of TonEBP mRNA is not present after 3 days of water deprivation. Interestingly, in both studies the urine osmolality was significantly higher in 3-day water deprived animals compared to water-replete animals. This is likely to be due to an increase in the corticomedullary osmotic gradient and, as a consequence, the tonicity of the renal interstitium in the papilla (Knepper, 1982; Knepper and Burg, 1983). In areas where the tonicity is highest, such as the inner medulla of rats, TonEBP mRNA is not affected by water deprivation despite the perceived increase in osmolarity of the interstitial fluid (Cha et al., 2001). Thus, hypertonicity may not necessarily lead to an increase in TonEBP mRNA abundance. Recently it was reported that hypertonicity in cultured renal cells resulted in an increase in TonEBP mRNA abundance, but the increase was due to the stabilization of the TonEBP mRNA pool rather than an increase in actual mRNA transcription (Cai et al., 2005).

In contrast to the effect of water deprivation on TonEBP mRNA transcription, 3 days of water deprivation in *N. alexis* resulted in an increase in the intensity of TonEBP-IR in the nuclei of the collecting duct cells in the outer and inner medulla and papilla. In addition,

the area immediately surrounding the nuclei of some of the collecting duct epithelial cells in the renal papilla appeared to have very little TonEBP-IR, suggesting that TonEBP had mostly translocated to the nucleus. These observations are also consistent with immunohistochemistry in water-deprived rats (Cha et al., 2001). In cultured cells, nuclear translocation of TonEBP is the typical response when the extracellular fluid is hypertonic (Woo et al., 2000b). Therefore, it appears that nuclear translocation of TonEBP can be induced without an increase in TonEBP mRNA abundance.

Unlike rats, desert rodents such as *N. alexis* can survive long-term water deprivation without suffering dehydration due to the production of metabolic water and a highly concentrated urine. Desert rodents, therefore, provide a unique opportunity to examine the role of TonEBP in renal function during long-term water deprivation. *N. alexis* subjected to long-term water deprivation (7 and 14 days) in this study showed an increase in the expression of TonEBP mRNA in the kidney. The increase in TonEBP mRNA may be due to an increase in mRNA stability as observed in cultured kidney cells (Cai et al., 2005), or an upregulation in the transcription of TonEBP mRNA. Nuclear translocation of TonEBP was also found in the kidney of 7- and 14-day water-deprived hopping mice, with the intensity of TonEBP-IR in the inner medulla and papilla being greatest after 14 days of water deprivation. This trend was particularly evident in the nuclei of the epithelial cells lining the collecting ducts in the papilla. The increase in TonEBP activity is consistent with the urine osmolality data for 7- and 14-day water-deprived hopping mice, which indicates that the cells in the papilla are bathed in extracellular fluid that has an equivalent osmolality to the urine (Knepper, 1982).

In addition to the renal papilla and inner medulla, the epithelial cells of the tubules in the outer medulla of *N. alexis* deprived of water for 7 and 14 days showed a greater intensity of TonEBP-IR than those of both the control and 3-day water-deprived hopping mice. This suggests that the interstitial fluid in this region of the kidney is hypertonic in comparison to that of control and 3-day water-deprived *N. alexis*, and that the corticomedullary osmotic gradient in the kidney of *N. alexis* may have increased during water deprivation.

In the renal cortex, the osmolality of the interstitium remains isotonic with the plasma, and previous studies in *N. alexis* have shown that plasma osmolality does not change in response to water deprivation (Heimeier and Donald, 2006). Rats deprived of water for 3 days showed no change in TonEBP-IR in the renal cortex (Cha et al., 2001). Similarly, TonEBP-IR in the cortex of *N. alexis* did not change in response to the different periods of water deprivation, which is consistent with the observations in rats (Cha et al., 2001).

Cells exposed to a hypertonic environment over a long period of time accumulate high concentrations of compatible osmolytes that, unlike inorganic ions and urea, do not inhibit intracellular proteins, enzymes or macromolecules (Kultz et al., 1998; Yancey et al., 1982). The increase in intracellular sorbitol, betaine, *myo*-inositol and taurine in renal cells exposed to hypertonic extracellular fluid has been shown to occur in response to an increase in the transcription of AR (Garcia-Perez et al., 1989; Smardo et al., 1992), BGT-1 (Nakanishi et al., 1990), SMIT (Kitamura et al., 1997; Kwon et al., 1992) and TauT (Ito et al., 2004), respectively. Furthermore, it is well established that TonEBP regulates the mRNA transcription of AR, BGT-1, SMIT and TauT by binding to the respective TonE consensus sites in their promoter regions (Woo and Kwon, 2002). Therefore, an upregulation of the transcription of AR, BGT-1, SMIT and TauT during water deprivation in *N. alexis*, will be an indication of the effect of water deprivation on TonEBP activity.

In *N. alexis*, 3, 7 and 14 days of water deprivation increased the mRNA expression of AR, BGT-1, SMIT and TauT mRNAs in the kidney. The mRNA data are supported by the immunohistochemical observation of nuclear translocation of TonEBP at each time point examined during water deprivation. These provide indirect evidence that the intracellular accumulation of sorbitol, betaine, *myo*-inositol and taurine is a key adaptation of the renal medulla of *N. alexis* in which the interstitial osmolality is likely to be between 5000 and 6000 mOsm after 14 days of water deprivation (Knepper, 1982). Thus, in desert rodents, TonEBP functionality is likely to be a key regulatory mechanism protecting renal cells from the extreme variations in medullary hypertonicity required to produce the highly concentrated urine necessary to survive in xeric environments.

LIST OF ABBREVIATIONS

AR	aldose reductase
BGT-1	betaine/GABA transporter
DAB	diaminobenzidine tetrahydrochloride
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
ORE	osmotic response elements
SMIT	*myo*-inositol transporter
TauT	taurine transporter
TonE	tonicity-responsive enhancer
TonEBP	TonE binding protein
TonEBP-IR	TonEBP immunoreactivity

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