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Osmoadaptation-related genes in inner medulla of mouse kidney using microarray

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Abstract

To distinguish biological molecular processes of osmotic stress occurring in inner medulla, we utilized microarrays to monitor expression profiles. RNAs from three segments (cortex, outer medulla, and inner medulla) of mouse kidney were isolated and applied to microarrays. We found 35 genes expressed highly in inner medulla. Next, microarrays for the RNAs from mouse medullary collecting duct cell line (mIMCD) cells and osmotically adapted mIMCD cells (HT cells) were performed (designed as resistant to 1270 mOsm/H_2O). Of 35 genes highly expressed in inner medulla, 6 genes such as; B-cell translocation gene protein (BTG), myc-basic motif homologue, gelsolin, cell surface glycoprotein, laminin β2, and tubulo-interstitial nephritis antigen, were also expressed highly in HT cells. Using real-time PCR, we confirmed the expression of six genes. Additionally acute osmotic stress induced the BTG. By comparing the inner medulla to a mIMCD3, we identified genes which respond to acute and chronic hyperosmotic stress.

Keywords: Microarray; Inner medulla; Osmotic stress

The major functions of the kidney are the excretion of waste or toxic substances and the regulation of water and salt content in the body. To accomplish this, the kidney has developed a specific organization, consisting of three structurally and functionally distinct zones, designated as cortex, outer medulla, and inner medulla [1]. Each region has specific functional characteristics such as nutrient reabsorption in the cortex and the urine concentration in the medulla. The cells in the inner medulla are particularly specialized, since they are not only effectors for concentrating the urine, but also deal with widely fluctuating concentrations of NaCl and urea. To survive and function in such a harsh environment, these cells have developed complex genetic programs enabling them to adapt to a hyperosmotic environment [2].

Whereas many functions occurring in specific renal tubule segments and regions have been described, the complex modalities of kidney function are still not completely understood. To characterize the molecular phenotypes of the kidney, a precise knowledge of the gene expression profile of the whole kidney would be of particular value. In addition, the detection of genes or gene families differentially expressed in the three kidney zones could point to specific mechanisms or pathways accounting for distinct renal features. Despite the importance of such basic knowledge, very few investigations have examined these questions [3–5].

The use of oligonucleotide microarray technology makes it possible to simultaneously evaluate the expression levels of thousands of mRNAs, thus representing

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an ideal tool for generating comprehensive information about organ physiology [6–8]. In the present study we used high-density oligonucleotide arrays from Affymetrix [9] containing 6520 probes (i.e., genes) to create a catalogue of the transcripts that are expressed in the three major regions of the mouse kidney. In addition, we compared these expression patterns to those observed in a chronic and acute osmotically stressed inner medullary collecting duct cell line (mIMCD3) to distinguish those genes most important for adaptation to osmotic stress. The results identify many known and novel biological processes that characterize the different zones of the kidney. Moreover, they demonstrate the utility of comparing in vitro cell culture models to in vivo tissues to define selected sets of genes that characterize specific biological processes.

Materials and methods

RNA isolation from tissue. Kidneys were removed from 6-week-old C57BL/6 male mice (The Jackson Laboratory) and sectioned into cortex, outer medulla, and inner medulla. Total RNA was isolated from the different regions using TRIzol Reagent (GIBCO/BRL).

Cell culture. Mouse inner medullary collecting duct-3 (mIMCD3) cells [10] were grown to confluence on plastic dishes (Falcon) in Dulbecco's modified Eagle's medium/Ham's F12 (1:1) (Life Technologies) supplemented with 10% fetal bovine serum (JRH Biosciences) and 2% penicillin–streptomycin (Life Technologies). For chronic adaptation to hyperosmolality, mIMCD3 cells at passage 12 were grown in culture medium, in which osmolality was increased approximately 100 mOsm every 48 h until 1270 mOsm was reached. Hyperosmolality was achieved by adding 1 ml of a stock solution containing equimolar concentrations of NaCl and urea to 10 ml culture medium. Accordingly, at the osmolality of 1270 mOsm (measured by freezing point depression; Advanced Instruments) the medium was supplemented with 320 mM NaCl + 320 mM urea. Cells adapted to hypertonic medium were designated as "HT cells." HT cells were maintained and passaged several times in the hypertonic medium until next experiments as a chronic hypertonic stress. Total RNA was isolated from control mIMCD3 and HT cells using TRIzol Reagent (Gibco/BRL, Rockville, MD). For acute hypertonic stress experiments, cells were exposed to hyperosmolar medium supplemented with 50 mM NaCl (100 mOsm) or 25 mM NaCl + urea 50 mM (100 mOsm), or urea 100 mM (100 mOsm) or 2× (200 mOsm), 3× (300 mOsm), and 4× (400 mOsm), respectively, for 3, 6, 12, 16, and 24 h. Total RNA was isolated and used for real-time PCR.

cDNA synthesis. cDNA was synthesized from total RNA using Superscript Choice kit (Gibco/BRL, Rockville, MD) and T7 polymerase (Mega Script T7 kit; Ambion, Austin, TX). Total RNA (8.0 μg) was annealed to T7-$(dt)_{24}$ primer (100 pmol/μl) at 70 °C for 10 min. Reverse transcription was carried out at 42 °C for 1 h in a mixture with final concentrations of 1× first-strand buffer, 10 mM dithiothreitol, 500 μM each dATP, dCTP, dGTP, and dTTP, and 20,000 U of Superscript II reverse transcriptase per ml, and the reaction was terminated by placing the tube on ice. Second-strand synthesis was carried out in 150 μl, incorporating the entire 20-μl first-strand reaction mixture and a 130-μl second-strand reaction mixture containing final concentrations of 1× second-strand buffer, 250 μM each dATP, dCTP, dGTP, and dTTP, 1.2 mM dithiothreitol, 65 U DNA ligase/ml, 250 U DNA polymerase I/ml, and 13 U RNase H/ml. The mixture was incubated at 16 °C for 2 h, whereupon 2 μl of T4 DNA polymerase at 5 U/μl was added and incubation at 16 °C was prolonged for 5 min. To terminate the reaction, 10 μl of 0.5 M EDTA was added. After purification, the cDNA was precipitated with 5 M ammonium acetate and absolute ethanol at −20 °C for 20 min. The pellet was resuspended in 1.5 μl RNase-free water.

Labeling by in vitro transcription. Synthesis of biotin-labeled cRNA was carried out by in vitro transcription using the MEGAscript T7 In Vitro Transcription Kit (Ambion, Austin, TX). The reaction was carried out at 37 °C for 5 h in a mixture with 7.5 mM ATP, 7.5 mM GTP, 5.6 mM UTP, 1.9 mM biotinylated UTP, 5.6 mM CTP, 1.9 mM biotinylated CTP, 1× T7-Transcription Buffer, and 1× T7-Enzyme Mix (Enzo Diagnostics, Farmingdale, NY). The amplified cRNA was purified with an affinity resin column (RNeasy, Qiagen).

Fragmentation, array hybridization, and scanning. The cRNA was fragmented by incubation at 94 °C for 35 min in the presence of 40 mM Tris–acetate (pH 8.1), 100 mM potassium acetate, and 30 mM magnesium acetate. The fragmented cRNA was hybridized to the GeneChip Murine Mu11K sub B Array Set (Affymetrix, Santa Clara, CA) allowing us to monitor the abundance of about 6500 mRNA transcripts representing known genes and ESTs. This array does not contain the 25% incorrect oligos that existed on a previous mouse GeneChip. A 220-μl hybridization solution of 1 M NaCl, 10 mM Tris (pH 7.6), 0.005% Triton X-100, 50 pM control oligonucleotide B2 (Affymetrix), control cRNA (Bio B 150 pM, Bio C 500 pM., Bio D 2.5 nM, and Cre X 10 nM) (American Type Tissue Collection, Manassas, VA, and Lofstrand Labs, Gaithersburg, MD), 0.1 mg of herring sperm DNA per ml, and 0.05 μg of the fragmented cRNA per μl was heated to 95 °C, cooled to 40 °C, and clarified by centrifugation before being applied to each of the four subarrays. Hybridization was at 45 °C in a rotisserie hybridization oven at 60 rpm for 16 h. Subsequent washing and staining of the arrays was carried out using the GeneChip fluidics station protocol EukGE-WS2. Briefly, the GeneChip probe arrays were washed 10 times at 25 °C with non-stringent wash buffer (6× SSPE, 0.01% Tween 20, and 0.005% antifoam). The second wash consisted of 4 cycles of 15 mixes per cycle with Stringent Wash Buffer [100 mM MES (its compound name; 2-*N*-morpholino ethanesulfonic acid, Sigma, St. Louis, MO), 0.1 M NaCl, and 0.01% Tween 20] at 50 °C. The arrays were stained for 10 min in streptavidin–phycoerythrin (SAPE) solution 1× MES solution, 0.005% antifoam, 10 μg/ml SAPE (Molecular Probes, Eugene, OR), and 2 μg/μl acetylated BSA (Sigma, St. Louis, MO) at 25 °C. The post-stain wash consisted of 10 cycles at 25 °C in the fluidics station. The probe arrays were treated for 10 min in antibody solution [1× MES solution, 0.005% antifoam, 2 μg/μl acetylated BSA, and 0.1 μg/μl normal goat IgG (Sigma, St. Louis, MO), 3 μg/μl antibody goat-anti-streptavidin, biotinylated (Vector Laboratories, Burlingame, CA)] at 25 °C. The final wash consisted of 15 cycles of 4 mixes per cycle at 30 °C in fluidics station. Following washing and staining, probe arrays were scanned 2 times at 3 μm resolution using the GeneChip System confocal scanner (Hewlett–Packard, Santa Clara, CA), controlled by GeneChip 3.1 software (Affymetrix).

Data analysis. The TEST 2 GeneChip (Affymetrix) was used to assess critical features of the mRNA preparations and the cDNA generated from the samples, and to evaluate the stringency of staining and hybridization. In addition, a battery of GeneChip controls was present on the TEST 2 GeneChip and on each of the Mu11K sub B GeneChips. These controls serve to normalize the fluorescence signal from each probe cell on each GeneChip and thus facilitate the reliable comparison of data from independent experiments.

Probe pairs representing genes such as GAPDH and β-actin serve as internal controls for monitoring RNA integrity and labeling fidelity. In addition, the probe arrays contain oligonucleotides representing sequences of three bacterial genes, BioB, BioC, BioD, and one phage gene, Cre, as quantitative standards. A twofold or greater change in average difference values from different kidney segments or different cell types, respectively, reflects real differences in gene expression, as stated in the manufacturer's instructions (Affymetrix). In addition, for each gene "Absolute Calls" were determined according to Affymetrix

algorithms and procedures [7,9]. The calculation of the ratio between perfect match (PM) to mismatch (MM) (PM/MM ratio) was used to define transcripts as present (P), marginal (M), or absent (A; undetected). We used the conservative default settings provided by Affymetrix for this calling.

Real-time PCR. We confirmed changes in five genes that appear to be related to the osmotolerance in inner medulla plus the housekeeping gene GAPDH using real-time one-step quantitative RT-PCR. The six genes included: glyceraldehyde-3-phosphate dehydrogenase (GAPDH), B-cell translocation gene-1 protein (BTG), gelsolin (GEL), cell surface glycoprotein A15 (A15), laminin β2 (LAN), myc-basic motif homologue-1 (MBH), and tubulo-interstitial nephrites antigen (TINA). Relative quantitation with real-time, one-step reverse-transcriptase polymerase chain reaction (RT-PCR) was performed with SYBR Green PCR Reagents and an ABI PRISM 7700 Sequence Detection System (PE Applied Biosystems, Foster City, CA), according to the manufacturer's instructions. Reactions were performed using 1.0 μl RNA at a concentration of 100 ng/μl, in a reaction volume of 50 μl. An RT reaction was performed at 48 °C for 30 min, followed by PCR consisting of AmpliTaq activation for 10 min at 95 °C, then 40 cycles with heating to 95 °C for 15 s and cooling to 60 °C for 1 min mRNA levels were normalized to levels of GAPDH. The PCR primers used were as follows:

GAPDH:
forward primer, 5′-CATGGCCTCCAAGGAGTAAG- 3′;
reverse primer, 5′-CCTAGGCCCCTCCTGTTATT-3′.
BTG:
forward primer, 5′-AGCCTGTGAGTCCAAGGAAA-3′;
reverse primer, 5′-TGACTGCGAAAGAGGGTTCT-3′.
MBH:
forward primer, 5′-TGATTCTCCAGCCTCCATCT-3′;
reverse primer, 5′-ATGTGTAGGCCTGGGTCTTG-3′.
GEL:
forward primer, 5′-CATCGAGACAGATCCAGCAA-3′;
reverse primer, 5′-AAAGGATCCACCGACCAGTA-3′.
A15:
forward primer, 5′-AAATGAGAAGGCCACCAATG-3′;
reverse primer, 5′-TGTCAGAAACATCGCGTACAG-3′.
TINA:
forward primer, 5′-CCTGTTCAAGCACTCATGGA-3′;
reverse primer, 5′-CCCACCCAGTGATCTTGACT-3′.
LAN:
forward primer, 5′-AAACTTCAACCGCCAACATT-3′;
reverse primer, 5′-CCACCTGTCCCTCTCCATAC-3′.

All primer sets were designed according to manufacturer's recommendations, and when feasible, to span one or more introns. Electrophoretic analysis of expected product sizes was performed for all primer sets prior to one step, real-time RT-PCR, to confirm the fidelity of the reaction.

Results and discussion

Gene expression profile of the whole kidney

Of the 6520 genes present on the microarrays, 2456 genes were expressed in one or more regions of the murine kidney. Among the 2456 genes expressed in the kidney 1751 transcripts (71.3%) were detectable in all three kidney zones (Fig. 1A).

The most abundant transcript in the whole kidney was prothymosin alpha (data not shown), a highly conserved

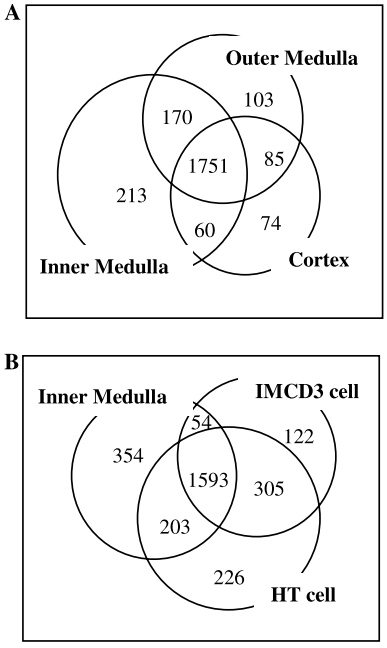

Fig. 1. (A) Numbers of genes expressed in the whole kidney, or in one, two, or all three kidney regions, respectively. (B) Numbers of genes expressed in the renal inner medulla, HT, and mIMCD3 cells, respectively.

protein, which is widely distributed among various tissues. The exact function of this protein is elusive, but it seems to be involved in immunomodulatory activities, thus conferring resistance to certain opportunistic infections [11]. Several of the other, highly abundant genes are also known to be involved in mechanisms protecting cells against the adverse effects of different stress factors such as oxidative stress, osmotic stress, or infection. For example, plasma glutathione peroxidase, phospholipid hydroperoxide, β-glutathione peroxidase, selenoprotein P, and sodium/potassium ATPase are involved in protection against oxidative stress [12–14], while stress proteins are related to osmotic stress [15]. The metalloproteinase-3 tissue inhibitor complexes with metalloproteinases and irreversibly inactivates them, a mechanism also contributing to the preservation of tissue from injury [16]. Thus, a major concern of kidney cells seems to be the defense against stressful situations.

According to the Affymetrix algorithm for Present and Abstract mRNAs, the largest number of expressed genes was detected in the inner medulla (2258) the lowest number in the cortex (1998) (data not shown). In particular, 74 genes (3.0%) were expressed exclusively in the cortex, and 103 (4.2%) exclusively in the outer medulla, but the highest number of region-specific genes, namely 213 (8.7%), was detectable in the inner medulla. This observation, as well as the fact that this kidney region contains the largest number of expressed genes, might be due to the exceptional physiological condition to which cells in the inner medullar are exposed. These

cells must deal with constantly changing extracellular osmolalities, generated by high urea and salt concentrations. To survive and function in such an extreme environment, the cells in this kidney zone have evolved a specialized genetic program that enables them to adapt to a hyperosmotic environment. Thus, the large number of genes specifically expressed in the inner medulla likely related to the many and diverse cell functions that are required in this region.

Gene expression profile of the renal inner medulla

Table 1 (A and B) shows the 35 genes with highest expression levels in the inner medulla, as compared to other regions. Genes coding for *hormones* or *hormone-related proteins*, *cell structure*, and *extracellular matrix* elements as well as genes involved in *metabolic processes*, *cell adhesion*, and the *stress–response* form the largest functional categories in this subset of genes. Within this group of genes, α-B2-crystallin showed the highest expression levels compared to other regions This gene is the major structural gene in the vertebrate ocular lens [17], but is also known to be highly expressed in the renal inner medulla. Its transcription is highly responsive to osmotic stress and, in this context, α-B2-crystallin was suggested to protect cells from the adverse effects of hypertonicity [18]. Similarly, we observed strong expression of heat shock protein 25 in the renal inner

Table 1
Genes overexpressed in inner medulla (IM)

Accession No.	Description	Classification	Average difference				
			C	OM	IM	HT	IMCD
(A) IM > C and OM, HT = IMCD							
AA108524	Aquaporin-CD	Cell surface receptors/transporters/proteins	466	558	1768	4	47
AA028501	Cytochrome *c* oxidase subunit VIII-H	Cytochrome *c* oxidase (COX)	64	52	186	28	27
Z48781	Stra1/Eplg2	Differentiation	99	77	304	22	28
X70853	MB-90/fibulin C form	Extracellular matrix and cell adhesion	−51	−14	102	36	74
M96827	Ob/ob haptoglobin	Heme-related	76	35	168	19	8
X95279	Spot14 gene	Hormones and related proteins	25	17	121	18	39
X59520	Cholecystokinin	Hormones and related proteins	−91	4	274	−70	−62
M13227	Enkephalin	Hormones and related proteins	27	38	116	24	17
X61397	Carbonic anhydrase-related polypeptide	Metabolism of carbohydrate and lipid	7	6	132	−8	−9
W29562	3T3-L1 lipid binding protein	Metabolism of carbohydrate and lipid	320	266	953	−8	−3
W09506	Fatty acid-binding protein	Metabolism of carbohydrate and lipid	134	86	566	−13	1
D00466	Apolipoprotein E gene	Metabolism of carbohydrate and lipid	19	14	222	−169	−237
Y10386	C1 inhibitor	Plasma proteins, immunoglobulins, coagulation	257	355	985	23	32
X04673	Adipsin	Plasma proteins, immunoglobulins, coagulation	410	115	988	31	61
AA049662	Plasma retinol-binding protein	Plasma proteins, immunoglobulins, coagulation	56	50	130	14	12
M64085	Serine proteinase inhibitor 2	Proteinase	−103	−106	105	6	4
AA097267	Plasma protease C1 inhibitor	Proteinase	252	237	530	196	184
X55123	GATA-3 transcription factor.	Transcription	49	55	149	−2	−10
D10024	Protein-tyrosine kinase substrate p36	Cell signaling	212	282	708	1303	1643
X56123	Talin	Cell structure/vesicle movement/secretion	48	42	240	86	229
X56304	Tenascin	Extracellular matrix and cell adhesion	41	40	168	30	77
X13135	Fatty acid synthase	Metabolism of carbohydrate and lipid	291	277	1058	548	1016
W29669	Interferon-related protein PC4	Differentiation	24	45	103	382	413
X91824	SPRR1a protein	Extracellular matrix and cell adhesion	67	106	618	670	422
X51971	Carbonic anhydrase	Metabolism of carbohydrate and lipid	27	33	148	86	119
AA034638	Heat shock protein 25	Stress protein	68	91	228	502	759
X53825	Heat stable antigen	Stress protein	427	634	1418	1550	1126
AA050733	Putative regulatory protein TSC-22	Transcription	−10	32	154	47	41
(B) IM > C and OM, HT > IMCD							
L16846	BTG 1	Cell cycle	158	250	610	1032	505
X54511	Myc-basic motif homologue-1	Cell structure/vesicle movement/secretion	33	63	219	940	449
J04953	Gelsolin	Cell structure/vesicle movement/secretion	161	203	480	722	249
AA050551	Cell surface glycoprotein A15	Cell surface receptors/transporters/proteins	53	58	196	332	24
U43541	Laminin β2 gene	Extracellular matrix and cell adhesion	48	25	131	160	36
AA137432	Tubulo-interstitial nephritis antigen	Extracellular matrix and cell adhesion	−2	0	122	313	99
M73741	α-B2-crystallin gene	Stress protein	287	548	2137	1465	931

(A) Genes overexpressed in IM (vs. outer medulla (OM) or cortex (C)). (B) Genes overexpressed in both IM (vs. OM and C) and HT cells (vs. IMCD cells).

(A) Genes overexpressed in IM more than twofold compared to outer medulla (OM) or cortex (C). (B) Genes overexpressed in both IM (compared to OM and C more than twofold) and HT cells (compared to IMCD cells more than twofold). Expression levels are shown as "average difference" as calculated using Affymetrix software. Relative change are calculated using Affymetrix software.

medulla, which also is known to be a medullary protein and to exhibit protective properties by stabilizing cytoskeletal elements [19,20]. In general, the modulation of the cells' architecture seems to be an important issue for inner medullary cells since several other genes important for cell structure (e.g., talin, gelsolin) the extracellular matrix and cell adhesion (e.g., fibulin, tenascin, collagen, laminin, and SPRR1) were highly expressed. This obvious requirement of genes involved in the modulation of cell structure is presumably related to the process of urine concentration, which evokes constantly changing extracellular solute concentrations in the inner medulla. As a first response to highly variable environmental osmolalities medullary cells either shrink or swell, both processes requiring significant reconstruction and resilience of cytoskeletal elements.

Comparison of gene expression profiles of the renal inner medulla and HT cells

To get a more detailed insight into the processes involved in the adaptation of inner medullary cells to hyperosmolality, and to distinguish genes specifically osmoresponsive, we evaluated the gene expression pattern of an osmotolerant mouse inner medullary collecting duct cell line [10]. Via chronic exposure of mIMCD3 cells to hypertonicity, achieved by an equimolar mixture of NaCl and urea in the culture medium, we generated a cell line capable of growing in a milieu with 1270 mOsm (designated as HT cells). Interestingly, as in the renal medulla, high amounts of stress proteins are detectable in these cells hinting at the activation of similar adaptive processes in vivo and in vitro [21]. On the whole 203 genes were detectable in the inner medulla and the HT cells, but not in the non-osmotically adapted control IMCD3 cells (Fig. 1B). Comparing the list of 35 genes highest expressed in the inner medulla (Table 1) to the genes highest expressed in HT cells, we found a small set of 7 genes highly expressed in both of these groups, but only weakly expressed the other groups investigated (Table 1B). This observation suggests that these transcripts play significant roles in osmoadaptation in vivo and in vitro.

To confirm the expression data generated by microarray technology, real-time quantitative RT-PCR was performed. The expression of those genes specific for inner medulla and HT cells (Table 1B) was analyzed: BTG 1 (BTG), myc-basic motif homologue-1 (MBH), gelsolin (GEL), cell surface glycoprotein A15, laminin β2 (LAN), and tubulo-interstitial nephritis antigen (TINA). The α-B2-crystallin was not analyzed because its role is already well studied in the inner medulla [18]. All of genes were confirmed to be significantly ($p < 0.05$) expressed highly in the inner medulla compared to cortex or outer medulla (Fig. 2A) and in the HT cells compared to IMCD cells (Fig. 2B), as the microarray data showed.

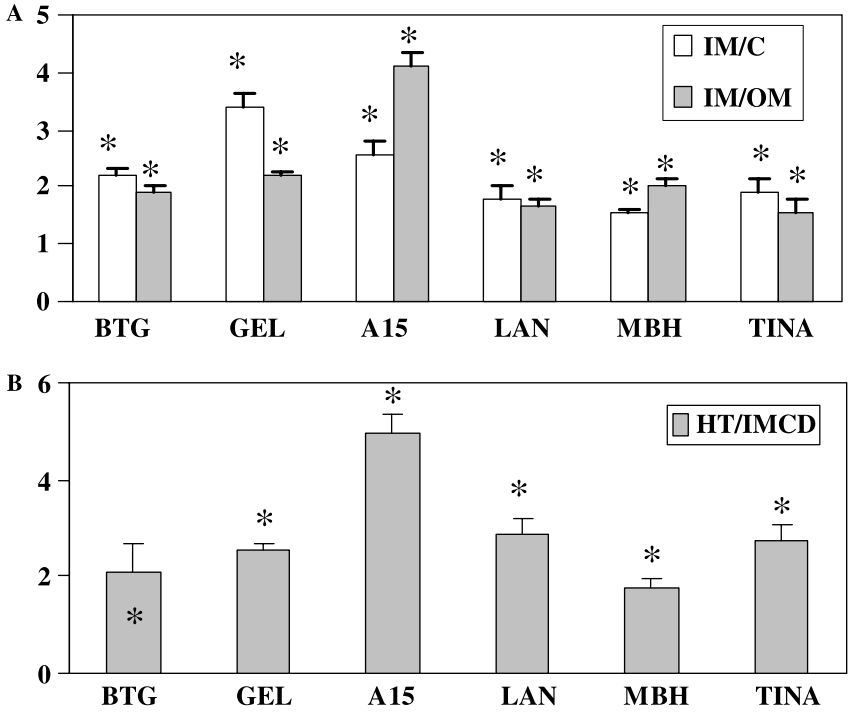

Fig. 2. Real-time RT-PCR to confirm microarray data of BTG 1 (BTG), gelsolin (GEL), cell surface glycoprotein A15 (A15), laminin β2 (LAN), myc-basic motif homologue-1 (MBH), and tubulo-interstitial nephritis antigen (TINA) expression. (A) Relative ratios of mRNA expression of each gene in inner medulla (IM)/cortex (C) and inner medulla (IM)/outer medulla (OM) are shown. (B) Relative ratios of mRNA expression levels of each gene of HT cells compared to mIMCD cells are shown. Data are shown by relative ratio. $n = 5$, $^*p < 0.05$ for each comparison.

And, when exposing mIMCD3 cells to acute osmotic stress, we observed a significant increase of BTG 1 expression (Fig. 3A). BTG 1 was induced by hypertonic stress by NaCl, which was observed by addition of urea alone. The peak of the expression of this gene was at 12 h after osmotic stimulation at more than at 600 mOsm (Figs. 3A and B).

It is intriguing to examine the known functional properties of the six transcripts increased by hyperosmotic stress. Of the six genes, BTG 1 is the only gene induced by both chronic and acute osmotic stress. BTG 1 transcript is a member of the BTG family that is associated with the early G1 phase of the cell cycle, and is known as an antiproliferative gene highly expressed in apoptotic cells [22,23]. Accordingly, this gene might be involved in reduction or inhibition of cell proliferation evoked by the inner medullary hyperosmotic environment. In fact, it is known that both, severe acute and chronic hypertonic stress, can slow down or even stop proliferation [23,24]. Besides, these results suggest that this transcript may protect the cells of the renal medulla against acute hypertonic stress induced apoptosis by the induction of G1 arrest of the cell cycle.

Myc-basic motif homologue-1 (MBH) and gelsolin are members of an actin filament severing and capping protein family that remodel the cytoskeleton in response to phosphatidylinositol 4,5-bisphosphate (PIP2) and Ca^{2+} during agonist stimulation [25]. Cell surface glycoprotein A15 exhibited highest expression levels in both, inner medulla and HT cells. It belongs to the tetraspanin family of proteins characterized by the presence of four conserved transmembrane segments and known to be involved in such diverse functions as cellular activation, adhesion, migration, and differentiation [26]. In contrast with BTG 1, this gene did not increase in acute hypertonic stress, although increased in chronic hypertonic

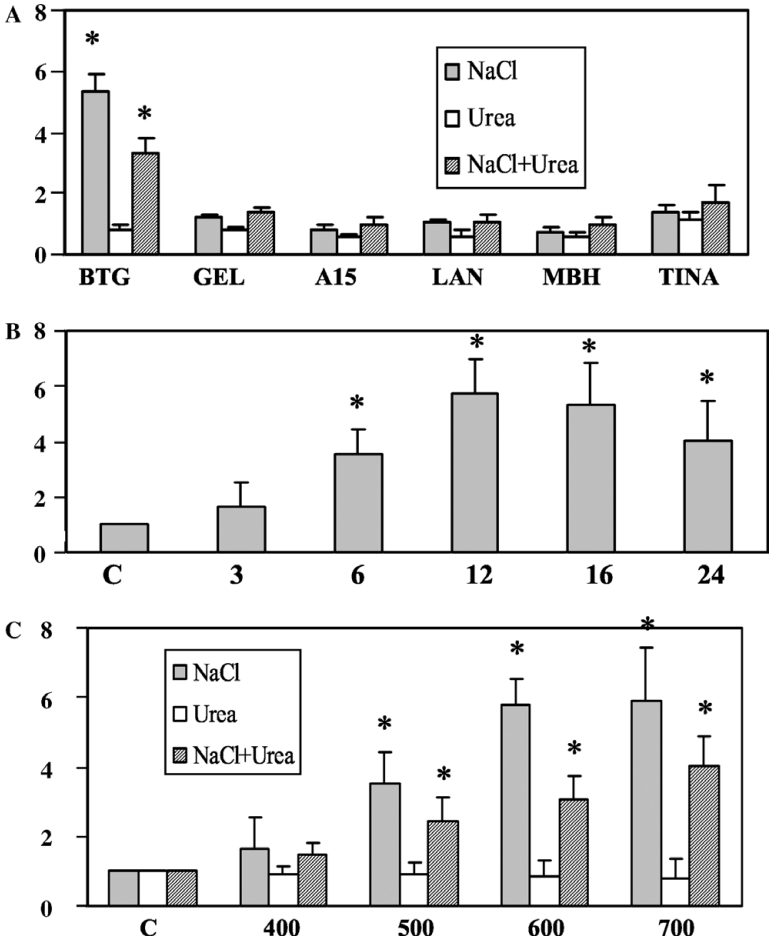

Fig. 3. Real-time RT-PCR to investigate to reaction for acute osmotic stress in the genes. (A) Relative ratios of mRNA expression of BTG 1 (BTG), gelsolin (GEL), cell surface glycoprotein A15 (A15), laminin β2 (LAN), myc-basic motif homologue-1 (MBH), and tubulo-interstitial nephritis antigen (TINA) of mIMCD3 cells exposed to acute osmotic stress compared to control are shown. mIMCD3 cells were treated with NaCl (+150 mM; 600 mOsm), urea (+300 mM; 600 mOsm), or both (NaCl +75 mM, +urea 150 mM; 600 mOsm) for 12 h. (B) Time course of BTG 1 mRNA expression levels in mIMCD3 cells exposed to acute osmotic stress with NaCl (+150 mM; 600 mOsm) for 0, 3, 6, 12, 16, and 24 h. (C) Alteration of BTG 1 mRNA expression of mIMCD3 cells exposed to various acute osmotic stresses (400, 500, 600, and 700 mOsm) using NaCl, urea or both for 12 h. Values are means ± SD; $n = 5$, $^*p < 0.05$ vs. control.

milieu. This may indicate that this transcript may play a role in adaptation to the hypertonic environment by differentiation and change of the cellular characteristic. Laminin is a component of basement membranes and is able to polymerize into a network that is cross-linked with type IV collagen [27]. Laminin β2 is a component of the basement membrane of the skeletal muscle neuromuscular junction, kidney glomerulus, nerve fascicle perineurium, and muscular smooth muscle [28–30], although the molecular function remains little known. Tubulo-interstitial nephritis antigen was reported to be localized at renal tubular basement membranes [31], and promote cell adhesion interacting with type IV collagen and laminin [32].

In general, the accumulation of transcripts related to *cell structure* elements, *extracellular matrix*, and *cell adhesion* processes points to the importance of the cytoskeleton for the correct functioning of cells in a stressful situation. Of note, it must be considered that HT cells are continuously growing in a hypertonic environment. Therefore, the pronounced expression of these genes is essential for the survival and the correct functioning of cells in a chronic hypertonic environment. From this observation it can be concluded that as in the inner medulla, these genes are necessary for osmoadaptive mechanisms and are not involved in rearrangement processes of cytoskeletal structures during cell swelling or shrinking.

Finally, the fact that inner medulla and HT cells have significantly more genes in common (203 genes) than inner medulla and non-osmotically stressed control IMCD3 cells (54 genes) points to physiological similarities between these biological states. This demonstrates the feasibility of comparing gene expression patterns of such different samples as in vivo tissue and in vitro cell culture material for the characterization of distinct biological processes.

In conclusion, comparing the expression pattern of the inner medulla, outer medulla, and cortex to an osmotically adapted mIMCD3 and normal mIMCD3, we identified 6 genes (BTG, GEL, A15, LAN, MBH, and TINA) highly responsive to chronic hyperosmotic stress, and one gene, BTG, is to acute hyperosmotic stress at in inner medulla.

Acknowledgments

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