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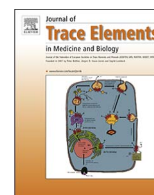

Physiology

Expression and immunolocalization of metallothioneins MT1, MT2 and MT3 in rat nephron☆

Ivan Sabolić[a,*], Mario Škarica[a,1], Marija Ljubojević[a], Davorka Breljak[a], Carol M. Herak-Kramberger[a], Vladiana Crljen[b], Nikola Ljubešić[c]

[a] Molecular Toxicology Unit, Institute for Medical Research and Occupational Health, Croatia
[b] Croatian Institute for Brain Research & Department of Physiology, School of Medicine, University of Zagreb, Croatia
[c] Croatian Academy of Sciences and Arts (HAZU), Zagreb, Croatia

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A B S T R A C T

Rodent kidneys exhibit three isoforms of metallothioneins (MTs), MT1, MT2 and MT3, with poorly characterized localization along the nephron. Here we studied in adult male Wistar rats the renal expression of *MTs* mRNA by end-point RT-PCR and MT proteins by immunochemical methods The expression pattern of *MT1* mRNA was cortex (CO) > outer stripe (OS) = inner stripe (IS) = inner medulla (IM), of *MT2* mRNA was IM > CO > IS = OS, and of *MT3* mRNA was IM > CO = OS = IM. MT1/2-antibody stained with heterogeneous intensity the cell cytoplasm and nuclei in proximal tubule (PT) and thin ascending limb, whereas MT3-antibody stained weakly the cell cytoplasm in various cortical tubules and strongly the nuclei in all nephron segments. However, the isolated nuclei exhibited an absence of MT1/2 and presence of MT3 protein. In MT1/2-positive PT cells, the intracellular staining appeared diffuse or bipolar, but the isolated brush-border, basolateral and endosomal membranes were devoid of MT1/2 proteins. In the lumen of some PT profiles, the heterogeneously sized MT1/2-rich vesicles were observed, with the limiting membrane positive for NHE3, but negative for V-ATPase, CAIV, and megalin, whereas their interior was positive for CAII and negative for cytoskeleton. They seem to be pinched off from the luminal membrane of MT1/2-rich cells, as also indicated by transmission electron microscopy. We conclude that in male rats, MTs are heterogeneously abundant in the cell cytoplasm and/or nuclei along the nephron. The MT1/2-rich vesicles in the tubule lumen may represent a source of urine MT and membranous material, whereas MT3 in nuclei may handle zink and locally-produced reactive oxygen species.

1. Introduction

Metallothioneins (MTs) are small (Mr = 6–8 kDa) cysteine rich proteins which bind Zn, Cd, and a few other toxic metals with variable affinity. In adult mammals, functional MTs exist in four isoforms. MT1 and MT2 (MT1/2) are widely expressed in different organs and in heterogeneous abundance [1], MT3 is found largely in brain, but also in a few other organs [2–6], whereas MT4 is found in some stratified squamous epithelia [7].

The exact functions of individual MTs have not been resolved. The expression of MT1/2, but not of MT3 and MT4, in the liver and other mammalian organs can be induced by a variety of stimuli, including some trace metals (Cd, Hg, Zn, Ag, Pt), glucocorticoids, physical stress, inflammation, starvation, irradiation, chemicals that produce oxidative radicals, *etc.* [8–24]. From these studies various roles for MT1/2 have been proposed, such as intracellular storage and homeostatic control of essential metals (Zn, Cu), absorption and/or excretion of some essential and toxic metals, detoxication of toxic metals by sequestration, scavenging of free radicals, protection against alkylating agents, and resistance to and/or protection from anticancer drugs. Other studies indicated poorly-defined roles of MT1/2 and MT3 in regulation of mitochondrial energy metabolism in mammalian organs, protection from DNA damage and apoptosis, regulation of gene expression during certain stages of the cell cycle, cell proliferation and differentiation, organs development, transepithelial ion and water transport, cancerogenesis and cancer diagnostics, and pathogenesis of some neurodegenerative diseases [5,6,16,24–30]. MT3 has also been studied as a growth inhibitory factor (GIF) for neurons and glia in human brain [29,31].

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* Corresponding author at: Molecular Toxicology Unit, Institute for Medical Research and Occupational Health, Ksaverska cesta 2, 10000 Zagreb, Croatia.
E-mail address: sabolic@imi.hr (I. Sabolić).
[1] Present address: Department of Neuroscience, Yale University School of Medicine, New Haven, CT, USA.

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Table 1
Primer sequences used in end-point RT-PCR studies of rat metallothionein genes.

Gene	Forward (F)/Reverse (R) Primers (5′-3′)	Accession No. Gene Bank	Location	RT-PCR product (bp)
rMT1a	F: CACCAGATCTCGGAATGGAC	NM_138826.4	56–75	220
	R: CAGCAGCACTGTTCGTCACT		275–256	
rMT2A	F: CACAGATGGATCCTGCTCCT	NM_001137564.1	86–105	249
	R: AAGTGTGGAGAACCGGTCAG		334–315	
rMT3	F: CCTGGATATGGACCCTGAGA	NM_053968.2	60–80	249
	R: AGGGACACGCAGCACTATTC		308–288	
rβ-Actin	F: GTCGTACCACTGGCATTGTG	NM_031144.2	518–537	364
	R: AGGAAGGAAGGCTGGAAGAG		862–881	

In the mammalian kidneys, besides in homeostatic regulation of essential metals, MTs may play a protective role in nephrotoxicity induced by Cd and other toxic metals [11,16,32,33]. In adult rats, by biochemical and immunochemical methods a limited abundance of MT1/2 proteins was found largely in cortical proximal tubules [10,16,34–40], whereas the MT3 protein was detected in some glomerular and collecting duct cells [6]. In the human kidney, MT3 was demonstrated in the cytoplasm of epithelial cells in glomeruli, cortical proximal and distal tubules, and collecting ducts [4].

An independent, detailed immunolocalization of various MTs along the mammalian nephron has not been performed. A recent observation in MT3-transfected cell line of the human proximal tubule, which exhibited formation of domes (not present in non-transfected cells), indicated that MT3 may be involved in the renal ion and water transport functions [5,41]. The MT3-transfected cells were also more sensitive to Cd-induced cytotoxicity [27,28]. These observations emphasized a need for a detailed characterization of MTs expression in various cell types along the mammalian nephron. In addition, a limited amount of MT1/2 has been detected in human and animal urine, but its origin has not been clarified; the urine MTs can be used as a biological marker of Cd exposure and Cd-induced renal dysfunctions [29,42–44]. In order to characterize the expression and localization of MTs in the rat kidneys, here we performed RT-PCR studies in various kidney zones of adult male rats, and immunochemical studies in tissue samples using the commercial (anti-MT1/2) and noncommercial (anti-MT3) antibodies. A possible expression of MT4 in the rat kidney was not a topic in this study, because previous mRNA studies indicated its absence in the mammalian kidneys [7,24].

2. Materials and methods

2.1. Animals, and human kidney

Male Wistar strain rats, 10–12 weeks old, from the breeding colony at the Institute for Medical Research and Occupational Health in Zagreb were used. Animals were bred and handled in accordance with the Directive 2010/63/EU on the protection of animals used for scientific purposes. Before and during experiments, animals had free access to standard laboratory food 4RF21 (Mucedola, Italy) and tap water.

Fresh tissue samples of human kidney cortex were obtained from the local hospitals in Zagreb. The samples were obtained from the adult male patiens that underwent surgical operations to remove tumors. Informed patient consent was obtained beforehand. The studies in rats and on human tissues were approved by the Institutional and hospital Ethic Committees.

2.2. Isolation of RNA, synthesis of first strand cDNA, and end-point RT-PCR

Rats were sacrificed under general anesthesia (Narketan, 80 mg/kg b.m. + Xylapan, 12 mg/kg b.m., i.p.; both from Chassot AG, Bern, Switzerland) by cutting large abdominal blood vessels and exsanguination under the stream of cold water. The kidneys were removed, decapsulated, rinsed in ice-cold saline, and the middle, ~1 mm-thick transversal tissue slice was immediately immersed into RNAlater solution (Sigma, St. Louis, MO, USA). The slice was later separated in morphologically distinctive zones (cortex, outer stripe, inner stripe, and inner medulla (papilla)) for RNA isolation. Total cellular RNA from these tissue zones was extracted using Trizol (Invitrogen, Karlsruhe, Germany) and cleaned using the RNeasy Mini Kit (Qiagen, Hilden, Germany) according to manufacturer's instructions. RNA concentration and its purity was tested spectrophotometrically at 260 and 280 nm (BioSpec Nano, Shimadzu, Japan). The quality and integrity of RNA was further checked by agarose gel electrophoresis, stained with StarGel (Lonza, Rockland Inc., ME, USA), and visualized under ultraviolet light. Isolated RNA was stored at −70 °C until use.

First strand cDNA synthesis was performed using the High Capacity cDNA RT Kit (Cat. #4374966; Applied Biosystems, Foster City, CA, USA) following the manufacturer's instructions. Total cellular RNA (1 μg) was incubated at 25 °C for 10 min in the reaction mixture containing random primers and reverse transcribed in total volume of 50 μL containing 1x reverse transcription buffer, 20 units of ribonuclease inhibitor, 1 mM of dNTP mix, and 40 units of Multiscribe reverse transcriptase, by incubation at 37 °C for 120 min and final denaturation at 85 °C for 10 min cDNAs were stored at −20 °C until use.

RT-PCR was performed in total volume of 20 μL using: 1 μL of 5x diluted first strand cDNA, 0.4 μM specific primers, and ready to use PCR Master Mix (Applied Biosystems) following instructions by the manufacturer. To avoid amplification of genomic DNA, intron over-spanning primers were used. Custom primers for the rat genes, *rMT1* (subtype *MT1a*), *rMT2* (subtype *MT2A*), *rMT3*, and *rβ-actin* were purchased online from Invitrogen (http://bioinfo.ut.ee/primer3-0.4.0/). The primer sequences are listed in Table 1. Reaction conditions used for PCR were the following: initial denaturation for 3 min at 94 °C, denaturation for 30 s at 95 °C, annealing for 30 s at 57 °C and elongation for 45 s at 72 °C. The non-template control, where the cDNA was substituted with DNase/RNase free water, exhibited no reaction (data not shown). RT-PCR products were resolved by electrophoresis in 1.5% agarose gel stained with 1xGelStar (Lonza, Rockland Inc., ME, USA), and visualized under ultraviolet light. The housekeeping gene *rβ-actin* was used to control variations in the cDNA input. The optimal number of PCR cycles within the exponential phase of the reaction was 20 for *rMT1*, 24 for *rMT2* and *rβ-actin*, and 33 for *rMT3*.

2.3. Antibodies and other material

Commercial monoclonal anti-MT antibody (clone E9; Code M0639, generated against self-polymerized equine MT1 and MT2), which recognizes a highly conserved domain common to mammalian MT1 and MT2 proteins (MT1/2-ab), was purchased from DAKO (Carpinteria, CA, USA). This antibody was previously used to study MT1/2 in human organs [24] and in the organs of intact and Cd-treated rats [16,45], Noncommercial polyclonal antibody to human MT3/GIF (MT3-ab) was previously characterized in immunocytochemical and Western analysis in human organs and cell lines [4,5,27,28,41,46]. In this study we also used noncommercial polyclonal antibodies for water channels AQP1

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