J. Trace Elem. Med. Biol. Vol. 17 (2) 97–106 (2003)
http://www.urbanfischer.de/journals/jtraceelm

Journal of
Trace Elements
in Medicine and Biology
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Influence of zinc deficiency on the mRNA expression of zinc transporters in adult rats

Michael W. Pfaffl[1,*] and Wilhelm Windisch[2]

[1] Institute of Physiology, Freising-Weihenstephan, Germany
[2] Department of Livestock Sciences, BOKU-University of Natural Resources and Applied Life Sciences, Vienna, Austria

Received March 2002 · Accepted January 2003

Abstract

The accumulation of zinc in the cell is a sum of influx and efflux processes via transporter proteins, like the four Zn transporters (ZnT1–4), the divalent cation transporter 1 (DCT1) and of storage processes mainly bound to metallothionein (MT). To study the effect of Zn deficiency on mRNA expression levels, adult rats were used as an animal model. Food intake was restricted to 8 g/day containing 2 µg Zn/g fortified with pure phytate in Zn deficiency rats and 58 µg Zn/g in controls (n = 7). At day 1, 2, 4, 7, 11, 16, 22, and 29 of Zn deficiency, 3 animals were sacrificed, respectively (n = 24). Zn deficiency was evident from reduced plasma Zn, plasma alkaline phosphatase activity and severe mobilization of Zn from tissue stores (mainly skeleton), while food intake and body weight remained unaffected. Tissues representing Zn absorption (jejunum, colon), Zn storage and utilization (muscle, liver), and Zn excretion (kidney) were retrieved. Total RNA contents increased in colon ($p = 0.003$) and trend to decrease in liver ($p = 0.086$). Zn deficiency was without effect on tissue total RNA concentrations in muscle tissue and kidney. Real-time reverse transcription (RT) polymerase chain reaction (PCR) assays were developed and a relative quantification on the basis of GAPDH was applied. Assays allowed a relative and accurate quantification of mRNA molecules with a sufficiently high sensitivity and repeatability. All known Zn transporter subtypes were found in the tissues. ZnT3 was newly elucidated and sequenced in rat tissues. Expression patterns and reactions to Zn deficiency were specific for the tissue analysed. Expression results imply that some transporters are expressed constitutively, whereas others are highly regulated in tissues responsible for Zn homeostasis. The most distinct changes of expression levels were shown in colon which can therefore be postulated as a highly Zn sensitive tissue. MT was down-regulated in all tissues, massively in liver ($p < 0.001$) and in colon ($p = 0.002$) and in tendency also in the jejunum and kidney. In parallel with intracellular Zn status it is a potent candidate gene for Zn deficiency. ZnT1 and ZnT2 showed a significant up-regulation of mRNA expression in colon ($p = 0.032$ and $p = 0.026$) and for ZnT2 a trend of down regulation in jejunum ($p = 0.098$). This study provides the first comparative view of regulation of gene expression and fully quantitative expression analysis of all known Zn transporters in a non growing adult rat model on a constant platform and therefore allows a direct comparison.

Key words: real-time RT-PCR; relative expression, rat, zinc transporter, zinc deficiency, ZnT1, ZnT2, ZnT3, ZnT4, DCT1, metallothionein

***Correspondence to:** Michael W. Pfaffl, Institute of Physiology, Weihenstephaner Berg 3, D-85354 Freising-Weihenstephan, Germany, Phone: + 49-8161-71-3511, Fax: + 49-8161-71-4204, E-mail: pfaffl@wzw.tum.de

0946-672X/03/17/02-097 $ 15.00/0

Introduction

Zinc is a small, hydrophobic, highly positive charged ion (Zn^{2+}), which can not cross biological membranes by passive diffusion. Therefore, specialized mechanisms are required in the organism for both the Zn uptake and the Zn release (1). These active transport processes via Zn-binding ligands require energy for Zn transport (2, 3) and their presence can significantly affect Zn transport into the cells (4). The importance of Zn in cell physiology is related mainly to its intracellular involvement into enzyme catalysis, protein structure, protein-protein interactions, and protein oligonucleotide interactions (3). The mechanisms by which Zn enters mammalian cells have been studied in a variety of cell systems: liver cells (5, 6), trophoblasten (7), fibroblasten (8), intestinal cells (9), endothelial cells (10) and intracellular membrane vesicles e.g. in the small intestine (2, 11, 12). The accumulation of Zn in the cell is a sum of influx and efflux processes via Zn transporter proteins, like the divalent cation transporter 1 (DCT1) and the four Zn transporters (ZnT1–4), and of storage processes bound to metallothionein (MT) (13). Zn homeostasis regulates Zn concentration in cells and tissues quite efficient and prevents the organism from excessive accumulation over a wide range of dietary Zn intake (14–18). Therefore, Zn is virtually non-toxic to the living organisms (19). Sequences of the above mentioned Zn transporters genes involved in mammalian Zn transport were recently cloned (20–23). They all predict proteins with multiple membrane spanning regions and histidine rich intracellular loops. All mammalian Zn transporters have tissue specific and cellular functions. ZnT1 was the first cloned, is located at the plasma membrane and functions as a Zn exporter in virtually all organs, especially in the small intestine and kidney (20, 25). ZnT2 may be associated with vesicular Zn uptake or cellular export in many organs, e.g. intestine, kidney and testis (21, 25). ZnT3 is responsible for intracellular vesicular Zn uptake in neurons and testis (22, 25) and seems to have the highest abundance in the brain. ZnT4 functions as an exporter and was detected at the plasma membrane of mammary gland and in the brain (23, 25). DCT1 is an metal transporter with affinity to iron, zinc, and other cations, that functions as a cellular importer for metal uptake and is located at the basolateral membrane in many organs, e.g. duodenum, jejunum, kidney and bone marrow (24, 25). DCT1 is mainly regulated by the tissue iron concentration (24). Despite the solid work describing the kinetics and characteristics of Zn transport, no protein directly associated with the Zn transport was described until recently (25). MT has a high Zn binding capacity, it is one of the strongest biological binding ligands for Zn and regulates the intracellular levels of free Zn through intracellular binding. MT gene expression is also regulated by cellular Zn concentrations and the close correlation between Zn and MT in tissues such as liver and pancreas is well documented (26, 27).

Compared to the general knowledge about the function of Zn transport proteins, there is only little information on their mRNA expression and regulation by Zn homeostasis except for some semi-quantitative studies on basis of Northern-Blot analysis (28) and densitometric analysis of RT-PCR (29). Therefore, it was the aim of this study to elucidate the effect of Zn deficiency on the expression of mammalian Zn transporters and storage proteins in various Zn absorbing tissues like jejunum and colon, in Zn storing tissues like muscle and liver, and in a excreting tissue, the kidney. The respective tissues were retrieved from a rat experiment (30), which represented a newly established animal model on Zn deficiency in adult individuals (31–33). We developed and validated various quantitative reverse transcription (RT) followed by polymerase chain reaction (PCR) assays and established them on a fully quantitative real-time platform (LightCycler, Roche Diagnostics, Mannheim, Germany). Real-time RT-PCR is a simple and sensitive method not only to detect, but also to measure even minute amounts of mRNA molecules. This offers important insights into the local mRNA expression of low abundant transcripts in various tissues (34). Thus, during the recent years, RT-PCR has become an increasingly useful tool for the mRNA quantification. Nowadays real-time RT-PCR using SYBR Green I® technology is more and more used to quantify physiologically changes in gene expression. It combines the ease and necessary exactness to be able to produce reliable and rapid results. Herein a relative quantification was applied (35, 36). The relative expression is based on the expression levels of a target gene versus a reference gene and adequate for most purposes to investigate physiological changes in gene expression levels (36).

Material and methods

Animal experiment

The rat tissues were retrieved from an animal model described in (30): 31 female, non-growing rats weighing 212 g were fed a purified, phytate-enriched diet at restricted amounts covering the energy requirement for maintenance (8.0 g per head and day). Dietary Zn remained either at its native level (2 µg/g, Zn deficiency) or was supplemented with $ZnSO_4$ at amounts covering the requirement of Zn (58 µg/g, control). 8 subgroups of animals (n = 3 respectively) were submitted to Zn deficiency for 1, 2, 4, 7, 11, 16, 22, or 29 days and then sacrificed. Control values were retrieved from 7 animals fed the control diet and sacrificed at the beginning of the study (day 0, n = 3) and at the end of the study (day 29, n = 4). Liver, colon, jejunum, muscle and kidney were removed immediately and shock-frozen in liquid nitrogen and then stored at –80 °C until total RNA extraction.

Total RNA extraction and reverse transcription

The total RNA extraction was performed with Trizol (Roche Diagnostics) according to the manufacturers instructions. Integrity of extracted total RNA was electrophoretically verified by ethidium bromide staining and by optical density (OD) absorption ratio $OD_{260\,nm}/OD_{280\,nm}$ >1.95. 1 µg of purified total RNA from sample preparation was reverse transcribed with 100 U of M-MLV Reverse Transcriptase RNase H^- Point Mutant Reverse Transcriptase (Promega, Madison, USA) using 100 µmol/L random hexamer primers (Promega) according to the manufacturers protocol.

Primer design

Primers were derived either from rat, human or mouse sequences, and designed to produce an amplification product which spanned at least two exons in the highly conserved coding region (CDS) of the appropriate coding sequence of multiple species. Therefore a multiple CDS alignment (*clustal alignment* in HUSAR® software) of the available mRNAs was done at DKFZ (37). Primer design and optimisation was done in the high homology regions of the multiple alignment with regard to primer dimer formation, self priming formation and primer melting temperature (HUSAR software at DKFZ). Primer sequences, the position in the coding region and the expected real-time RT-PCR product length, and exon spanning region are summarized in Table 1. Primer for MT are able to amplify the transcripts of subtype 1 and subtype 2 MT mRNA. For ZnT2 and ZnT3 no information about the intron/exon structure are available in the published sequence databases EMBL (38) and GenBank (39).

Table 1. Forward (for) and reverse (rev) primer sequences (5'– >3'), their position in the coding sequence in rats (rn), mouse (mm) or human (h), RT-PCR product length and exon spanning of the investigated factors: zinc transporter 1 (ZnT1), ZnT2, ZnT3, ZnT4, divalent cation transporter 1 (DCT1), metallothionein (MT), housekeeping and reference gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH). For ZnT2 and ZnT3 no intron/exon structure is available either in human, rat or mouse sequences (NM012890, AF067405, NM003459, NM011773, AF067405).

Primer	Sequence (5'– > 3')	Position and Length	Exon spanning
GAPDH for GAPDH rev	GTC TTC ACT ACC ATG GAG AAG G TCA TGG ATG ACC TTG GCC AG	rn/h 301–497 197 bp	3 – >5
ZnT1 for ZnT1rev	GAC CAG GAG GAG ACC AAC A GAA AAG TAG AAG ACC AAG GCA TT	rn 562–824 263 bp	1 – >2
ZnT2 for ZnT2 rev	CCA GTG TCC GAG CTG CCT T GAT GGA GAA GAG GAA GGT GC	rn 609–745 137 bp	–
ZnT3 for ZnT3 rev	TTT TCA CAG AGC CCT CAG AG GCA TCA GTC ATG ATG GCC A	h/mm 98–317 220 bp	–
ZnT4 for ZnT4 rev	TTC CCA CTC CCT GCC TTC AAA TT AGC ACA CCA ACA CTT TGT ACC A	rn 741–872 132 bp	4– >5
DCT1 for DCT1 rev	CTG AGG AGG AGT ACT CTT GTT TGG GAC CTT GGG ATA CTG A	rn 173–435 263 bp	3 – >5
MT for MT rev	CTC CTG CAA GAA GAG CTG CT TCA GGC GCA GCA GCT GCA CTT	rn/h 81–186 106 bp	2 – >3

Real-time RT-PCR

For each investigated transcript a master-mix of the following reaction components was prepared to the indicated end-concentration: 6.4 µL water, 1.2 µL $MgCl_2$ (4 mmol/L), 0.2 µL Forward Primer (0.4 µmol/L), 0.2 µL Reverse Primer (0.4 µmol/L) and 1.0 µL LightCycler Fast Start DNA Master SYBR Green I (Roche Diagnostics). 9 µL of the master-mix was filled in the glass capillaries and 1 µL volume, containing 25 ng reverse transcribed total RNA, was added as PCR template. Capillaries were closed, centrifuged and placed into the LightCycler rotor. To improve the SYBR Green I quantification, a high temperature fluorescence measurement point was performed (40). The temperature for the elevated fluorescence acquisition in the 4th segment is listed in Table 2. It melts the unspecific PCR products at the elevated temperature, e.g. primer dimers, eliminates the non-specific fluorescence signal and ensures an accurate quantification of the desired product. The following real-time PCR protocol was used for amplification: *denaturation program* (10 min at 95 °C), a *four-segment amplification and quantification program* repeated 40 times (factor-specific amplification conditions with a single fluorescence measurement are summarized in Table 2), *melting curve program* (60 °C to 99 °C with a heating rate of 0.1 °C/s and continuous fluorescence measurements), and finally a *cooling program* down to 40 °C. For the described mathematical model (34, 35) it is necessary to determine the crossing points (CP) for each transcript. In this study *"second derivate maximum method"* was performed for CP determination, using the LightCycler Software, Version 3.5, 2001 (Roche Molecular Biochemicals).

Relative quantification

The relative expression ratio of a target gene is computed, based on real-time PCR cross-

Table 2. LightCycler real-time PCR cycling conditions of all seven assays: *Denaturation program* (10 min at 95 °C), a *four segment amplification and quantification program* repeated 40 times with a single fluorescence measurement, *melting curve program* (60 °C to 99 °C with a heating rate of 0.1 °C/s and continuous fluorescence measurements) and finally a *cooling program* down to 40 °C.

	assay steps (segments)	duration (s)	investigated transcripts						
			GAPDH	MT	ZnT 1	ZnT 2	ZnT 3	ZnT 4	DCT 1
I	Denaturation	15	95	95	95	95	95	95	95
II	Primer annealing	10	60	60	60	60	60	60	60
III	Elongation	20	72	72	72	72	72	72	72
IV	Fluorescence acquisition	5	84	86	84	82	88	83	86

ing point (CP) deviation (ΔCP) of a unknown sample versus a control (Fig. 1):

$$\text{expression ratio} = \frac{2^{(\Delta CP\ \text{target gene})}}{2^{(\Delta CP\ \text{reference gene})}} \text{ (equation 1).}$$

The concept of the threshold fluorescence is the basis of an accurate and reproducible quantification using fluorescence based RT-PCR methodologies (41). Threshold fluorescence is defined as the point at which the fluorescence rises appreciably above the background fluorescence, indicated in Fig. 1. Herein the *"second derivate maximum method"* was used for CP determination, where CP will be measured at the maximum increase or acceleration of fluorescence, even if the fluorescence levels between curves are different (41). A linear relationship between the CP, crossing the threshold fluorescence, and the log of the start molecules input in the reaction is given (42, 43). Therefore quantification will always occur during the exponential phase, and it will not be affected by any reaction components becoming limited in the plateau phase (33).

In the used mathematical model (equation 2) the target gene expression (herein MT and Zn transporters) were intra-sample normalised by a non regulated reference or housekeeping gene (34) like glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (44–46). GAPDH is present in all nucleated cell types since they are necessary for basis cell survival. The mRNA synthesis of this gene is considered to be stable and secure in various tissues, even under experimental treatments (44-46).

$$\text{expression ratio} = \frac{2^{(\Delta CP\ \text{MT})}}{2^{(\Delta CP\ \text{GAPDH})}} \text{ (equation 2).}$$

Statistical analysis

Total RNA concentrations per mg tissue of each single animal (n = 31) were calculated as function of Zn depletion days [d] and plotted as linear regression:

total RNA concentration = intercept + [slope * d].

GAPDH normalised relative expression data of each tissue and transcript (n = 31) on basis of 25 ng reverse transcribed total RNA were calculated as function of [d] and plotted as linear regression:

relative expression = intercept + [slope * d].

Statistical comparisons were performed by using the Sigma-Stat software V 2.0 for Windows 95 (Jandel Scientific Software, San Rafael, CA, USA). Expression data groups passed the Normality Test and Constant Variance Test. Correlation of tissue RNA content and expression data were shown by correlation coefficient (r). Data pairs of variables with positive correlation coefficient and $p < 0.05$ tended to increase together, and were regarded as significant. A trend of significance was defined for $0.05 < p < 0.10$.

Results

Confirmation of primer and RT-PCR product specificity

Specificity of the desired products in rat tissues were documented with high resolution gel electrophoresis (Fig. 2) and additionally with melting curve analysis. Derived melting temperature of RT-PCR products determined by melting curve analysis were transcript specific. The ZnT3 RT-PCR product was sequenced (MWG Biotech, Ebersberg, Germany) and newly elucidated in *Rattus norwegicus* and published in the EMBL and GenBank (Ac. No. AJ458940). 81.8% and 95.0% homology to the known human and mouse sequences were found.

Total RNA in investigated tissues

Fig. 3 presents the time course of total RNA concentration in two rat tissues. Zn deficiency was without effect in muscle tissue and kidney, but tended to change RNA contents of liver (decrease), jejunum (increase) and colon (increase) after about 1 week of Zn depletion. Expressed as a linear correlation to the duration of Zn deficiency, the increase was statistically evident in colon (r = +0.516, $p = 0.003$). A trend of decreasing total RNA concentration was determined in liver (r = −0.314, $p = 0.086$).

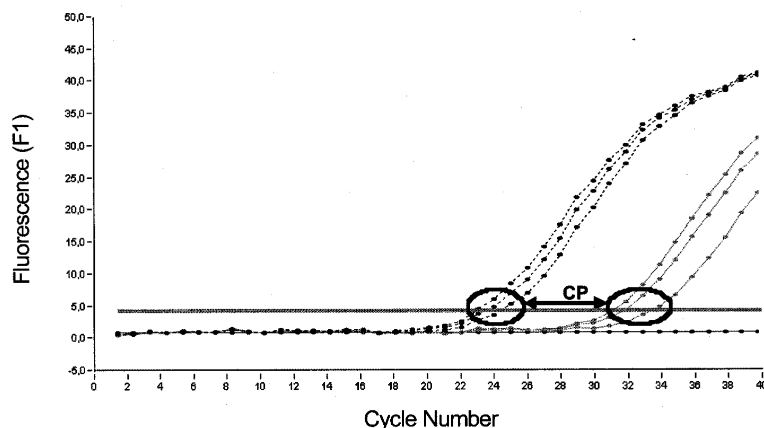

Fig. 1. Quantitative MT real-time RT-PCR fluorescence history on LightCycler platform. Crossing point differences (ΔCP) indicate extremely different expression levels of MT mRNA in Zn deficiency rat liver compared to controls, calculated according to equation 2. Real-time RT-PCR kinetics of control animals are shown in black dotted lines (---●---) and Zn deficiency rats are shown in grey solid lines (—●—). The negative water control is the black bottom line (—●—). Background fluorescence is indicated by blank solid grey lines (——).

Mean expression levels of investigated genes in the organs

Real-time RT-PCR detected Zn transporters and MT in all analysed rat tissues (liver, colon, jejunum, muscle and kidney). The raw expression levels (average values of all investigated samples) are presented in the order of magnitude either for the respective types of mRNA within tissues (Table 3) or for the abundance of a specific type mRNA among tissues (Table 4). Tissues exhibited an individual expression pattern for Zn storage and transport proteins. In all tissues, especially in muscle tissue, the GAPDH expression was dominant, followed by the ZnT4 expression. ZnT3 was very low abundant in colon, muscle and kidney. ZnT1, ZnT2, DCT1 and MT had tissue specific mRNA abundance levels.

Expression of the housekeeping gene GAPDH

GAPDH was used as reference and housekeeping gene in order to compare the quantified mRNA molecules of the MT and Zn transporters in the relative expression ratio model (equation 2). The dominant GAPDH expression showed no significant regulation under Zn deficiency in all investigated tissues or groups. Additionally, its variation was quite low: liver 3.8%, jejunum 5.8%, colon 8.6%, muscle 5.4% and kidney 7.8%.

Fig. 2. High resolution 4% agarose gel electrophoresis of all zinc transporter and GAPDH real-time RT-PCR products derived from rat liver total RNA. **Lane 1 + 18:** length standard (2 kbp, 1.2 kbp, 800 bp, 400 bp, 200 bp, 100bp); **lane 3–16 (duplicates):** MT (106 bp), DCT1 (263 bp), ZnT1 (263 bp), ZnT2 (137 bp), ZnT3 (220 bp), ZnT4 (132 bp), GAPDH (197 bp).

Relative changes in mRNA expression of MT and Zn transporters due to Zn deficiency

The RNA expression levels of MT and Zn transporters relative to GAPDH varied considerably with respect to type of tissue and duration of Zn deficiency. Table 5 shows the regulative response of MT and Zn transporter RNA expression to Zn deficiency, calculated as relative changes between the extreme treatment groups (control group versus Zn deficiency group suffering 29 days of Zn deple-

Table 3. Absolute ZnT1, ZnT2, ZnT3, ZnT4, DCT1, and MT expression levels in five different rat tissues.

tissue	absolute expression levels
jejunum	GAPDH > ZnT4 > ZnT2 > MT > DCT1 > ZnT3 > ZnT1
colon	GAPDH > ZnT4 > ZnT1 > MT > DCT1 > ZnT2 > ZnT3
liver	GAPDH > ZnT4 > ZnT1 > MT > ZnT2 > ZnT3 > DCT1
muscle	GAPDH ≫ ZnT4 > ZnT2 > ZnT1 > DCT1 > ZnT3 > MT
kidney	GAPDH > ZnT4 > MT > ZnT1 > ZnT2 > DCT1 > ZnT3

Table 4. Order of specific transcript mRNA expression abundance in rat tissues.

transcript	expression abundance in tissues
GAPDH	muscle > > liver > kidney > colon > jejunum
MT	kidney > liver > jejunum > colon > muscle
ZnT1	liver > kidney > muscle > colon > jejunum
ZnT2	jejunum > kidney > liver > colon > muscle
ZnT3	liver > muscle > kidney > jejunum > colon
ZnT4	liver > muscle > kidney > colon > jejunum
DCT1	liver > muscle > jejunum > kidney > colon

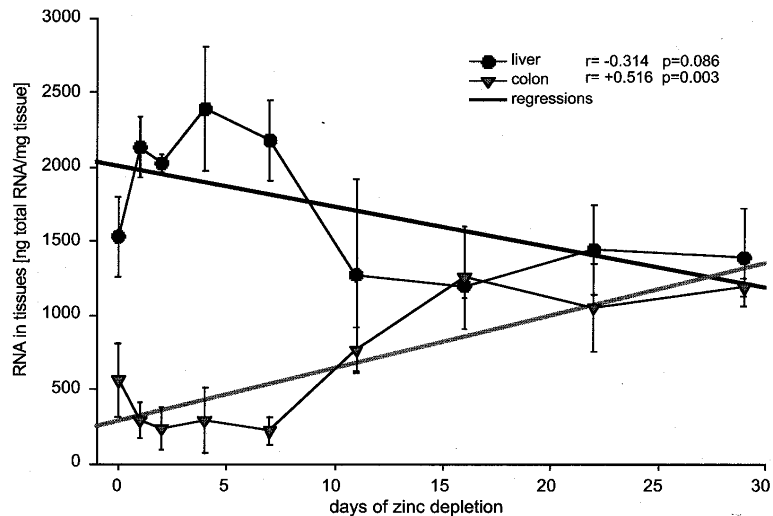

Fig. 3. Mean (Zn deficiency n = 3, control animals n = 7) of total RNA concentration [ng total RNA/mg tissue] in liver and colon of rat suffering 29 days of zinc deficiency (r = regression coefficient; *p*-value of regression).

Table 5. Relative expression of MT, ZnT1, ZnT2, ZnT3, ZnT4, and DCT1 mRNA normalised by the reference gene GAPDH mRNA expression. Numbers indicate the n-fold up- (↑) or down- (↓) regulation of the extreme groups: 29 days zinc depleted group in comparison to control group. *p*-values and correlation coefficients (r) of linear regressions were calculated by Sigma-Stat software. Significant differences ($p < 0.05$) and trends of regulations ($0.05 < p < 0.10$) are indicated in **bold letters**.

	MT	ZnT 1	ZnT 2	ZnT 3	ZnT 4	DCT 1
jejunum	↓1.39	↓3.79	↓5.56	↑8.04	↑1.24	↑1.27
	***p* = 0.062**	*p* = 0.241	***p* = 0.098**	*p* = 0.345	*p* = 0.635	*p* = 0.154
	r = −0.339	r = −0.217	r = −0.303	r = +0.195	r = +0.088	r = +0.262
colon	↓6.74	↑10.06	↑1.39	↑9.29	↑2.09	↓5.53
	***p* = 0.002**	***p* = 0.032**	***p* = 0.026**	*p* = 0.277	***p* = 0.003**	*p* = 0.245
	r = −0.532	r = +0.386	r = +0.399	r = +0.201	r = +0.512	r = −0.215
liver	↓154.7	↑1.04	↓1.71	↓1.03	↑1.13	↓1.88
	***p* < 0.001**	*p* = 0.895	*p* = 0.172	*p* = 0.988	*p* = 0.901	*p* = 0.885
	r = −0.558	r = +0.075	r = −0.256	r = −0.009	r = +0.011	r = −0.101
muscle	↓1.28	↓1.55	↓3.04	↓9.98	↓1.20	↓1.26
	p = 0.842	*p* = 0.883	*p* = 0.263	*p* = 0.271	*p* = 0.879	*p* = 0.789
	r = −0.037	r = −0.105	r = −0.207	r = −0.207	r = −0.092	r = −0.114
kidney	↓1.27	↓4.57	↑1.07	↑4.49	↓1.18	↑2.56
	***p* = 0.093**	*p* = 0.328	*p* = 0.668	*p* = 0.335	*p* = 0.682	***p* = 0.064**
	r = −0.307	r = −0.182	r = +0.081	r = +0.177	r = −0.076	r = +0.336

tion). Data are shown as n-fold up- (↑) or down- (↓) regulation and the respective correlation coefficients.

MT was down-regulated in all tissues, massively in liver 154.7-fold ($p < 0.001$) and in colon 6.74-fold ($p = 0.002$) and in tendency also in the jejunum and kidney.

ZnT1 and ZnT2 showed a significant up-regulation of mRNA expression in colon ($p = 0.032$ and $p = 0.026$) and for ZnT2 a trend of down regulation in jejunum ($p = 0.098$). ZnT2 expression ranged at 10^4-times less in muscle, 10^3-times less in colon and about 100-times less in liver, kidney and jejunum compared to the GAPDH expression level.

ZnT3 mRNA showed changes in the expression levels but no significance could be elucidated due to very low expression levels. For ZnT4 an two fold up-regulation could be elucidated in colon ($p = 0.003$). DCT1 had a trend of 2.56-fold up-regulation in kidney ($p = 0.064$).

Fig. 4 and Fig. 5 present in detail the respective data for MT and ZnT2 in a logarithmic scale. MT was expressed at rates of about 10^4-times less in muscle, 10^2-times less in colon and jejunum, 10-times less in liver and kidney compared to the constant reference gene expression of GAPDH. The Zn deficiency treatment reduced MT expression progressively in all analysed tissues except the muscle. The respective correlation coefficients between MT expression and duration of Zn deficiency were highly significant in liver and colon.

Discussion

The aim of the study was to establish a sensitive system to quantify the low abundance gene expression of Zn transporters and storage proteins as well as to quantify them in physiological relevant tissues. Comparative expression studies between tissues and different zinc transporters can be done due to the usage of a constant platform and identical intra-sample normalisation procedure using GAPDH. Up to now investigations on the protein level are impossible, because proper antibodies for Zn transporters in rats are only available for ZnT1 and ZnT4. Therefore we have designed, optimised and validated six real-time RT-PCR assays of factors involved in the action of Zn transport on a constant platform. RT-PCR amplification of derived PCR products was shown to be sensitive, with high precision and reproducibility. Amplification of genomic DNA was avoided by primer pairs located on different exons (except ZnT2 and ZnT3), therefore a DNAse treatment of tissue total RNA samples was not necessary. A great simplification for the determination at the mRNA level of the parameters was achieved by use of a relative quantification strategy and a simple mathematical model (35).

The physiological status of the animals was characterized by the absence of growth and a constant food intake matched to the maintenance requirement of energy (36). As reported elsewhere (47) metabolic markers as well as blood plasma levels of growth related hormones including the expression levels of their receptor proteins remained unchanged during the entire experiment. Consequently, there was no interaction between Zn deficiency *per se* and the metabolism *in toto*, as it is usually the case in an animal model based on fast growing individuals (e.g. hormonal disorders due to standstill of growth, depression of food intake). Nevertheless, Zn deficiency was evident from a negative Zn retention, the quantitative Zn mobilization from storage tissues (mainly skeleton) and severely reduced plasma Zn concentrations and alkaline phosphatase activities especially at the end of the study (30). The present animal model may thus be considered as to represent the effect of Zn deficiency on mRNA expression of MT and Zn transporters in adults living at maintenance level (in a neither anabolic nor catabolic situation), as it is the most common case in humans, like in earlier studies (28, 29).

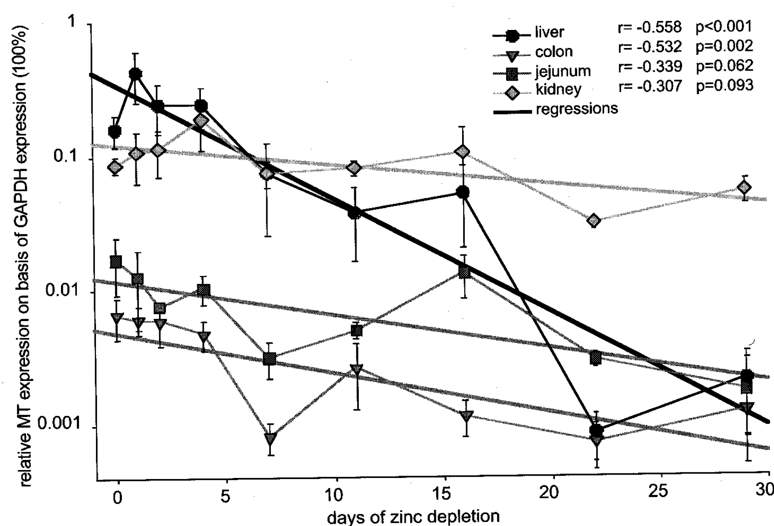

Fig. 4. Relative MT expression on the basis of GAPDH expression (=1.0). The exponential relationship in liver, colon, jejunum and kidney of zinc deficiency and MT mRNA expression is illustrated by the linear regression mean values on logarithmic scale (r = Pearson correlation coefficient; *p*-value of correlation).

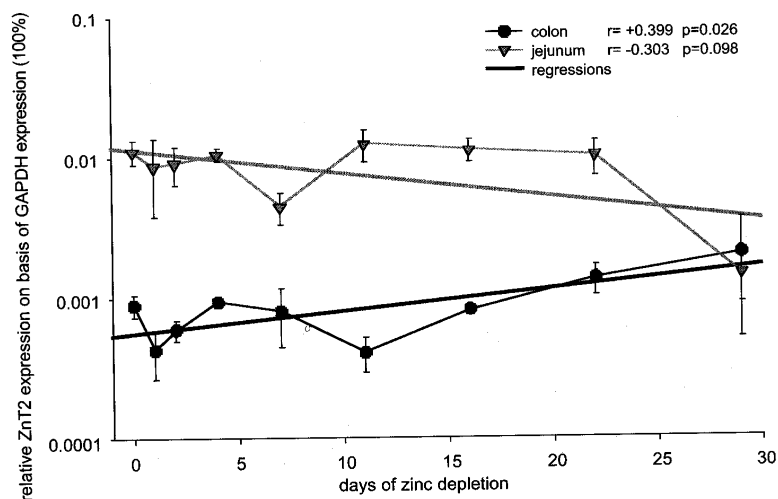

Fig. 5. Relative Zinc transporter 2 (ZnT2) expression on the basis of GAPDH expression (= 1.0). The exponential relationship in colon and a tendency in jejunum of zinc deficiency and ZnT2 mRNA expression is illustrated by linear regression mean values on logarithmic scale (r = Pearson correlation coefficient; *p*-value of correlation).

Zn depletion affected total RNA contents of all tissue types. Increased total RNA concentrations per weight could be observed in colon and a trend of decreasing concentration in liver. Jejunum, liver and kidney exhibited constant RNA contents. As shown in earlier publications, Zn deficiency may generally decrease DNA, RNA and protein (e.g. alkaline phosphatase) content per weight in rat tissues (30, 48). In the present study however, the depressive effect on total RNA seemed to arise not before about one week after the onset of Zn deficiency (Fig. 3). This delay in reaction corresponded to the observation that the quantitative Zn metabolism attempted to maintain regular Zn fluxes at the expense of short-term mobilisable Zn stores, which were largely exhausted after about one week of Zn deficiency (30). Obviously, the cellular contents of total RNA reflect the metabolic availability of Zn in terms of filling levels of tissue Zn stores.

Herein the MT expression of MT subtype 1 and 2 were measured simultaneously. Minimally 14 different human MT genes are already located in a gene cluster of about 82 kb on chromosome 16 (14, 49), testifying to the complexity of the MT gene family. Only the MT subtypes 1, 2, 3 and 4 will be translated to the corresponding Zn active binding proteins. For primer design, MT-1 and -2 were the best candidates, because they are expressed in a lot of tissues (50) with available sequences for several species. As known, MT

mRNA expression was down-regulated (26, 27, 51) or trend to be regulated in all investigated tissues, except in muscle. This leads to the hypothesis that MT expression might be a sensitive marker on the expression level for Zn deficiency. Expression of the mouse MT-1 gene in response to Zn is regulated by the Zn finger transcription factor MTF-1 (52, 54). MT mRNA expression was down-regulated in all investigated tissues and showed a quick response to Zn deficiency within a few days (Fig. 4 and Table 5). The described down-regulation of MT mRNA due to Zn deficiency is also known from other studies (26, 27, 50, 51, 52). In total, the expression of MT mRNA in tissues seems to be a sensitive transcription marker of Zn deficiency.

The investigated Zn transporters were expressed in jejunum, colon, liver, muscle and kidney and were regulated tissue specificly under the Zn depletion treatment. With the established sensitive real-time RT-PCR all transcripts could be quantified in all tissues. ZnT3 was newly elucidated in *Rattus norwegicus* and the sequence is published in GenBank and EMBL. Herein no limitation due to the applied method and resulting quantification sensitivities were derived in comparison to earlier publications working with Northern-Blot (28) or semi quantitative RT-PCR (29). In the first study a comparative response of ZnT-1, 2 and 4 to dietary Zn was examined in rats. ZnT-1 and ZnT-4 were expressed ubiquitously, whereas ZnT-2 expression was limited to small intestine, kidney, placenta and, in some cases, to the liver (28). The expression of ZnT-1 and ZnT-2 was comparable to Zn-induced changes in MT mRNA levels, suggesting a similar mode of regulation for these genes. The relative difference in regulation by Zn was reported there: ZnT-2 > ZnT-1 > ZnT-4 (28). A second semi-quantitative study (29) indicated that the Zn transporter expression is developmentally regulated in the islets of pancreas of growing rats. All Zn transporters were expressed in liver but only ZnT1 was shown to be existent during development. In contrast, herein it could be shown, that ZnT4 is the major transcript and is more abundant than ZnT2, ZnT1 or the very low abundant ZnT3. A new expression order of the mean expression levels could be shown in Table 3, quantified on a constant platform and using a similar intra-sample normalisation GAPDH procedure.

For Zn absorption, the small intestine plays the key role (53), represented in this study by the jejunum. When the small intestine is impaired, also caecum and colon can participate in Zn absorption (54). Each of the transport systems for Zn influx and efflux were individually regulated in the intestinal tract. In colon the most changes in the expression levels could be shown and therefore it can be postulated as a very Zn sensitive tissue. ZnT4 and ZnT2 or in some cases ZnT1 are the major transcripts in the absorptive colon, responsible for Zn efflux in the cell (25). These transcripts are all significantly up-regulated under Zn deficiency, which leads to a better Zn absorption for the organism under Zn deficiency (55). On the other hand DCT1, responsible for Zn influx, seemed to be down-regulated. In jejunum we found the inverse situation of Zn transporter 1, 2 and 4 expression. The role of MT in the absorption process in the gastrointestinal tract, particularly in conjugation with the Zn transporters, is still unclear (56).

In liver and muscle tissue, no regulation of the Zn transporters could be shown, as published earlier for ZnT1 (28, 55). This observation might lead to the assumption that the Zn supply to liver and muscle tissue is of high priority due to vital functions, while the other analysed tissues (especially from the intestine) are involved more into regulation and maintenance of a sufficient Zn flux towards the liver and muscle. In the kidney, the Zn transporters were regulated divergently. Only DCT1 expression trends to be up-regulated in kidney. The urinary Zn excretion is low and not changing during Zn deficiency (30), suggesting that Zn re-absorption in the kidney is not affected by Zn concentrations. MT concentrations in the kidney trend to be lower in rats suffering from Zn deficiency.

For ZnT3 mRNA a constant expression level, without any obvious regulation, could be postulated in all investigated tissues. The investigated tissues inhibit high variations in mRNA concentrations over the treatment period on a very low abundant level. It seems that ZnT3 is up-regulated in the absorptive jejunum and colon, and down-regulated in muscle. As described previously (22) very high ZnT3 mRNA concentrations were found in all rat brain regions: cerebellum, hypothalamus, left and right cortex (own data not published).

Conclusion

This study provides the first comparative view of gene expression regulation and fully quantitative expression analysis of all known Zn transporters (57), including ZnT3, in a non-growing adult rat model. In view of the data provided, the RT-PCR assay developed herein allows a relative and accurate quantification of Zn transporters and MT mRNA molecules with a sufficiently high sensitivity even for tissues with low mRNA abundance. The expression results indicate the existence of Zn transporter subtypes in various rat tissues, their different expression pattern and their tissue specific regulation under Zn deficiency treatment. The results show that all transporters and MT have unique expression patterns. The colon is a very Zn sensitive tissue in view to the found expression results. Expression results imply that some transporters are expressed constitutively, whereas others are highly regulated in tissues responsible for Zn homeostasis. In all tissues the MT mRNA expression level reflects the intracellular Zn status best. In comparison to the control group, MT mRNA was down-regulated in all tissues. MT subtype 1 and 2 mRNA expression is a potent candidate as marker gene for Zn deficiency. In further studies, the abundance and functionality of the Zn transporters should be investigated on the protein level.

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