Influence of zinc deficiency on the mRNA expression of zinc transporters in adults rats

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INTRODUCTION

The accumulation of zinc (Zn) 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).

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 and densitometric analysis of RT-PCR. 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 the excreting tissue like the kidney. The respective tissues were retrieved from a rat experiment, which represented a newly established animal model on Zn deficiency in adult individuals.

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. 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.

ANIMAL EXPERIMENT

The rat tissues were retrieved from an animal model described earlier (1): 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 (1.4 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 (each n = 3) were submitted to Zn deficiency for 1, 2, 4, 7, 11, 16, 22, or 29 days and then euthanised. Baseline values were retrieved from animals fed the control diet and euthanised at day 0 (n = 3) and day 29 (n = 4) of the study. Liver, jejunum, colon, muscle and kidney were removed immediately after euthanising and total RNA was extracted.

$$R = \frac{(E_{target\ gene})^{\Delta CP_{target\ gene}(mean\ control\ -\ mean\ sample)}}{(E_{GAPDH})^{\Delta CP_{GAPDH}(mean\ control\ -\ mean\ sample)}}$$

RELATIVE mRNA QUANTIFICATION METHOD

Herein a relative quantification was applied. The relative expression is based on the expression levels of a target gene versus a reference gene and adequate for the most purposes to investigate physiological changes in gene expression levels. Expression studies were done in real-time RT-PCR and each sample was normalised to the internal GAPDH expression *(= housekeeping gene).* Relative expression levels of Zn deficiency group and control group were compared with the corresponding control group, which were set to 1.0. The relative expression ratio (**R**) was calculated in real-time RT-PCR from the PCR efficiencies (**E = 2**) and the mean crossing point (**CP**) deviation (**ΔCP**) of the unknown sample group mean versus the control group mean (2).

Figure 1: High resolution 4% Agarose gel electrophoresis.

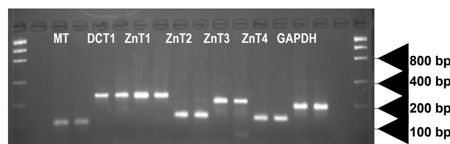


Table 1: Relative expression of target genes normalised by GAPDH expression. Numbers indicate the n-fold UP- (↑) or DOWN- (↓) -regulation of the extreme groups: 29 d zinc depleted group in comparison to control group.

	MT	ZnT 1	ZnT 2	ZnT 3	ZnT 4	DCT 1
jejunum	↓ 1.39 p = 0.062 r = 0.339	↓ 3.79 p = 0.241 r = 0.217	↓ 5.56 p = 0.098 r = 0.303	↑ 8.04 n.d.	↑ 1.24 p = 0.635 r = 0.088	↑ 1.27 p = 0.154 r = 0.262
colon	↓ 6.74 p = 0.002 r = 0.532	↑ 10.06 p = 0.032 r = 0.386	↑ 1.39 p = 0.026 r = 0.399	↑ 9.29 n.d.	↑ 2.09 p = 0.003 r = 0.512	↓ 5.53 p = 0.245 r = 0.215
liver	↓ 154.7 p < 0.001 r = 0.558	↑ 1.04 n.d.	↓ 1.71 p = 0.172 r = 0.256	↓ 1.03 n.d.	↑ 1.13 n.d.	↓ 1.88 n.d.
muscles	↓ 1.28 p = 0.842 r = 0.037	↓ 1.55 n.d.	↓ 3.04 p = 0.263 r = 0.207	↓ 9.98 n.d.	↓ 1.20 n.d.	↓ 1.26 n.d.
kidney	↓ 1.27 p = 0.093 r = 0.307	↓ 4.57 p = 0.328 r = 0.182	↑ 1.07 p = 0.668 r = 0.081	↑ 4.49 n.d.	↓ 1.18 p = 0.682 r = 0.076	↑ 2.56 p = 0.064 r = 0.336

Figure 2: Relative MT expression on the basis of GAPDH expression.

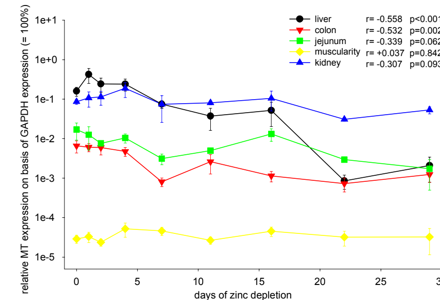
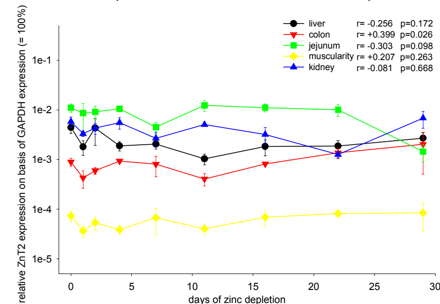


Figure 3: Relative ZnT2 expression on the basis of GAPDH expression.



RESULT & DISCUSSION

GAPDH mRNA expression

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. 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%, muscularity 5.4% and kidney 7.8%.

Absolute expression levels of investigated genes and organs

Real-time RT-PCR detected Zn transporters and MT in all analysed rat tissues: liver, jejunum, colon, muscle and kidney (figure 1). All tissues exhibited an individual expression pattern for Zn storage and transport proteins. In all tissues, especially in muscularity the GAPDH expression was dominant, followed by the ZnT4 expression. ZnT3 was very low abundant in colon, muscularity and kidney. ZnT1, ZnT2, DCT1 and MT had tissue specific mRNA abundance levels.

Relative changes in mRNA expression 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 1 shows the regulative response of MT and Zn transporter RNA expression to Zn deficiency, calculated as relative changes between the extreme animal groups [baseline control versus 29d suffering from Zn deficiency [up- (↑) or down- (↓) regulation] and the respective correlation coefficients of changes in expression during the time sequence of Zn deficiency. MT was down-regulated in all tissues, massively in liver, colon and in tendency also in the jejunum and kidney. ZnT1 and ZnT2 showed a significant up-regulation of mRNA expression in colon and for ZnT2 a trend of down regulation in jejunum . 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. DCT1 had a trend of 2.56-fold up-regulation in kidney. Figure 2 and 3 presents in detail the respective data for MT and ZnT2 in a logarithmic scale. MT was expressed at rates of about 10^{-4}-fold (muscle), 10^{-2}-fold (colon and jejunum) and 0.1-fold (liver, kidney) compared to GAPDH. Zn deficiency 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. ZnT2 expression ranged at 10^{-4}-fold (muscle), 10^{-3}-fold (colon) and about 10^{-2}-fold (liver, kidney, jejunum) of the GAPDH level. Zn deficiency did not seem to induce major changes in ZnT2 expression of tissues except for an increase in the colon.

CONCLUSION

This study provides the first comparative view of gene expression regulation and fully quantitative expression analysis of all known Zn transporters in a non-growing adult rat model. In view of the data provided the developed 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. Colon is a very Zn sensitive tissue in view to the 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 MT expression level reflects the intracellular Zn status best. In comparison to control group MT mRNA was down-regulated in all tissues. MT subtype 1 and 2 mRNA expression is a potent candidate as a marker gene for Zn deficiency.

(1) Windisch, W. (2002) Trace Elem. Med. Biol. 2002; 17 (submitted).

(2) Pfaffl, M.W. (2001) Nucleic Acids Res. 2001; 29: 2002-2007.