Identification of genes with altered expression in zinc-deficient rats by use of DNA microarrays

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Introduction

Zinc is an essential trace element with a large number of health related functions. It is a cofactor in numerous enzymes and various metabolic pathways, plays a role in hormone secretion and participates in immune functions. In turn, zinc deficiency causes anorexia, growth retardation, skin lesions, disturbed immune response and altered taste perception [1/2]. Besides its catalytic and structural functions, zinc plays also a role in regulation of gene expression. Whereas the symptoms of severe zinc deficiency in mammals are well known, a reliable diagnosis of a marginal zinc-supply is still not possible [3]. We have therefore explored whether characteristic changes in gene expression can serve as potential biomarkers in the assessment of the body zinc status. As a starting point we analysed the global changes in gene expression in selective zinc deficiency [4/5] in rats force fed with a zinc-deficient diet. Gene expression profiles have been determined for liver tissue of zinc-adequate (Zn+) and zinc-deficient (Zn-) rats by use of two different array systems (Fig. 1).

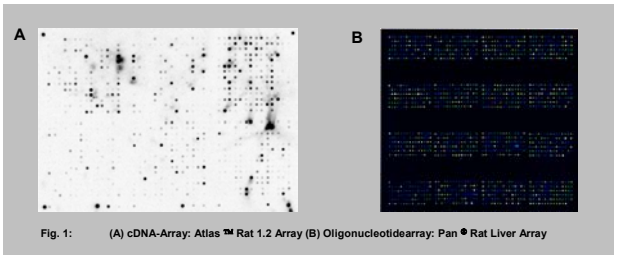

Fig. 1: (A) cDNA-Array: Atlas ™ Rat 1.2 Array (B) Oligonucleotidearray: Pan ® Rat Liver Array

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Methods

Generation of zinc-deficiency *in-vivo* and RNA-isolation. 24 male Sprague-Dawley rats (∅=121 g) were divided into 2 groups of 12 rats each. All rats were force-fed a semisynthetic diet (AIN 93G) by intragastric tube four times per day. The control diet contained 25 mg Zn/kg diet, the depletion diet 1.3 mg Zn/kg. At day 11 of the experiment all rats were killed by decapitation after a light anesthesia with diethyl ether. Blood for biochemical analyses was collected into heparinised tubes. Samples of tissues of interest were collected, shock-frozen in liquid nitrogen and stored at –80 °C for RNA isolation. Total RNA was isolated from liver for both types of arrays using modified single-step guanidium thiocyanat / acidic phenol / chloroform extraction methods.

Processing of cDNA- und oligonucleotide-arrays. For screening of differentially expressed genes in zinc deficiency cDNA-Nylon-Arrays Atlas™ Rat 1.2 Arrays (CLONTECH, Palo Alto) with 1176 single spotted cDNAs and Pan® Rat Liver oligonucleotidarrays with 1353 gene specific 50mer oligonucleotides (MWG Biotech, Ebersberg) on glass slides were employed. In both cases synthesis of gene specific [α- ^{33}P]-labeled resp. fluorescence-labeled (Cy3/Cy5) cDNA-probes was performed according to the manufacturers protocols. Four cDNA membranes for each dietary condition were separately but synchronously hybridized with the [α- ^{33}P]-labeled cDNA-probes. Hybridisation at 68 °C was allowed to proceed for 18 h, after which the membranes were washed and exposed to the phosphor imager screen for 96 h. Quantitative analysis after densitometric measurement of gene and array specific parameters was carried out by comparison of signal intensities of spots from zinc-adequate and zinc deficient states. The normalisation procedure included the reference to intensities of control genes as well as the sum of the intensities method. For oligonucleotide-arrays, the hybridisation of Cy5/Cy3-probe mixtures was carried out for 20 h at 42 °C in hybridisation chambers on 4 different slides. After the following washes the arrays were scanned (Affymetrix® 428™ Array Scanner) under dried conditions. ImaGene™ 4.2-Software (BioDiscovery, Inc.) was utilized for quantitative analysis. If signal intensities ratios Zn-/Zn+ differed by factors of 1.8 or 0.5, genes were considered as „zinc-dependent".

Conformation of expression changes by Northernblot and quantitative Real-Time RT-PCR. An independent conformation of differential expression of select array-identified candidate genes was performed by the Northern capillary blotting method. PCR-products from ORF´s of genes to be verified served as templates for [α- ^{32}P]-probe synthesis. Additionally some genes were proved by Real-Time RT-PCR (LightCycler, Roche). Based on sequence information provided by CLONTECH gene specific primers were synthesized. For online-detection during Real-Time-PCR-analysis the intercalating dye SYBR-Green was used. Melting curve and native gel electrophoresis served for characterisation of final products.

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Results I

The selective zinc-deficiency could be established by the lowered serum zinc concentration, reduced activity of serum alkaline phosphatase and increased mRNA levels of the marker gene metallothionein in liver tissue (Fig. 2).

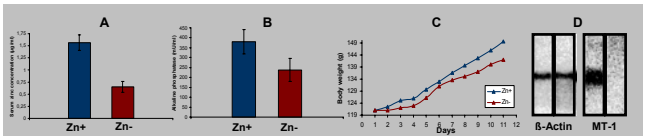

Fig. 2: (A) Serum zinc concentration (B) Activity of serum-AP (C) Weight gain (D) Metallothionein 1 mRNA-levels in zinc-adequate and zinc-deficient rats

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Results II

By use of cDNA-arrays and oligonucleotide-arrays 62 „zinc-dependent" genes could be identified. For 31 selected genes an independent conformation of altered mRNA-levels was performed by northern blotting. The differential expression of 23 genes was independently confirmed whereas for 5 genes no confirmation was possible. Transcripts of the residual 3 genes were below the level of detection by northern blot analysis. Additional 5 genes identified were reexamined by Real-Time RT-PCR. Table 1 lists the top ranking genes from which 42 were down-regulated and 22 showed upregulation and the proposed functions of the corresponding proteins. Northern blot analysis and quantitative PCR confirmed 84 % of the cDNA array-generated data and 89 % of those obtained by the oligonucleotide-arrays. Fig. 3 shows selected findings from the northern blot analysis.

*Table 1: Zn-dependent changes in gene expression in rat liver (* = Atlas ™ Rat 1.2 Array, ** = Pan ® Rat Liver Array; [a] = Northern Blot, [b] = quantitative Real-Time RT-PCR)*

Gene	GenBank Acc.#	Δ fold in deficiency	Verification	Function
Lipid metabolism				
3-keto thiolase acyl-CoA	M32801 *	-2,17 x		Fatty acid degradation
delta2, delta3 enoyl-CoA isomerase	D00729 **	-1,82 x	verified [a]	Fatty acid degradation
Carboxylesterase	AF171640 **	-1,75 x	verified [a]	Fatty acid degradation
ATP-citrat lyase	J05210 **	2,29 x		Fatty acid synthesis
Glycerol kinase	D16102 *	1,61 x	verified [a]	Fatty acid synthesis
Fatty acid transport protein	U89529 *	-2,63 x		Fatty acid uptake
Lysophospholipase	D63885 *	-3,45 x		Phospholipid degradation
Colipase precursor	M58370 *	-2,56 x	verified [a]	Triglyceride degradation
Protein metabolism				
Ubiquitin-like protein	AF095740 **	1,79 x	reverted [a]	Protein degradation
Acyl-peptide hydrolase	J04733 **	-5,26 x	verified [a]	Protein degradation
Serine dehydratase	Y00752 **	-1,85 x	verified [a]	Amino acid degradation
Cytosolic aspartate aminotransferase	D00252 **	-1,56 x	verified [a]	Transamination
Energy turnover				
SU of F0F1-ATP Synthase	D13127 **	1,8 x	verified [a]	ATP synthesis
Alcohol dehydrogenase A SU	M15327 *	-1,89 x	verified [a]	Ethanol oxidation
5-aminoevulinate synthase	J03190 **	-2,27 x	verified [a]	Porphyrine synthesis
Ceruloplasmin	L33869 *	2,10 x		Fe(II) oxidation
similar NADPH Ubiquinone	**	1,71 x		Electron transfer
Xenobiotic metabolism / stress response				
Cyp4A locus, cytochrome P-450 (IVA3)	M33936 * ; **	-2,13 x	verified [a]	Phase I enzyme
NADPH-cytochrome P450 reductase	M12516 *	-2,13 x	verified [a,b]	Phase I enzyme
Cytochrome P-450 2C23	X55446 *	-1,75 x		Phase I enzyme
Liver aldehyde oxidase	AF110477 **	-1,64 x	verified [a]	Xenobiotic metabolism
Metallothionein-1 und-2	M11794 **	-12,5 x	verified [a]	Stress response
AP endonuclease	D44495 *	1,76 x		DNA repair
Glutathione S-transferase subunit 5 theta	X67654 *	3,81 x		Detoxification (ROS)
similar glutathione transferase	**	2,18 x		Detoxification (ROS)
Hormon metabolism				
Insulin-like growth factor binding protein, compl. acid-labile SU	S46785 *	-1,41 x	verified [a]	Growth
Insulin-like growth factor binding protein 1	M89791 *	-2,50 x	verified [a,b]	Growth
Insulin-like growth factor binding protein 2	J04486 *	-2,33 x	verified [a,b]	Growth
Epidermal growth factor receptor	M37394 *	-1,82 x	verified [a]	Growth
Hepatic product spot 14	K01934 **	2,09 x	verified [a]	Thyroid answer
Neuropeptide Y	M20373 *	-1,92 x	verified [a,b]	Neuropeptide
Interleukin 2	M22899 *	-4,55 x		Autokr. growth factor
Trafficking / signal transduction				
Syntaxin 4	L20821 *	-2,13 x	verified [a]	Trafficking
Epimorphin, Syntaxin 2	L20823 *	-2,78 x		Trafficking
Brain fatty acid-binding protein	U02096 *	2,76 x		Trafficking
Synapsin II	M27925 *	-3,13 x	verified [a]	Trafficking
Ras-related protein	J02998 *	-2,17 x		Signal transduction
P2U purinoreceptor 1	U09402 *	-1,89 x		Signal transduction
G protein coupled receptor	U65417 *	2,70 x		Signal transduction
Prostaglandin F2 alpha-receptor	U47287 **	1,86 x		Signal transduction
B2 bradykinin receptor	L26173 *	2,34 x		Signal transduction
Cathepsin H	M36320 *	1,82 x		Intracell. signal peptidase
Arrestin D	U03629 *	-2,00 x	verified [a,b]	Receptor activity regulator
Others				
Transcriptional repressor NAB1	U17253 **	-1,64 x		Transcriptional control
Galactose-1-phosphate uridyl transferase	L05541 **	1,67 x		Carbohydrate metabolism
Plasma proteinase inhibitor alpha-1-inhibitor III	J03552 *	1,95 x		Acute phase protein
Voltage gated K+ channel protein	M59980 *	-3,3 x		Excitation signal conduction
Steryl-sulfatase	U37138 *	-1,81 x		Steroid hormon activator

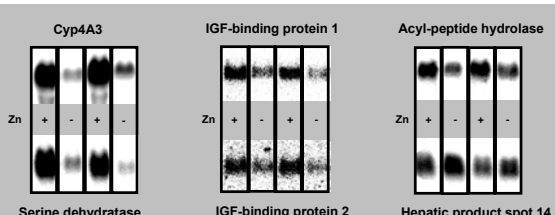

Fig. 3: Conformation of identified genes by northern blot-analysis (total-RNA from liver tissue of zinc- adequate and zinc-deficient rats (10 µg/Lane))

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Summary & Conclusions

For identification of „zinc-dependent" genes *in-vivo* a selective zinc-deficiency was established in rats. Changes in mRNA-expression in liver tissue were investigated using DNA-Array technology. 62 candidate genes with altered mRNA-levels could be identified. The zinc-dependent modulation of gene expression was verified for 23 genes by northern blot analysis and/or Real-Time RT-PCR. The corresponding gene products play a role in metabolism of lipids, proteins, hormones and xenobiotics as well as in stress response, trafficking, signal transduction and energy turnover. Corresponding to previous observations on growth retardation in zinc deficiency [6] many transcripts identified relate to the growth hormone and growth factor axis.

In conclusion, the array technology is suitable as a screening system for identifying a limited number of possibly regulated genes, but independent methods have to be employed additionally for verification and quantitation of the effects.

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References

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