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Expression Profile of Hepatic Metallothionein-I and ATP7B, and Brain Metallothionein-III and Acetyl cholinesterase Genes in Wistar Rat Model for Non-Wilsonian Brain Copper Toxicosis

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Abstract

Cognitive waning due to chronic copper (Cu) intoxication in animal models is increasingly being reported; notwithstanding, information regarding molecular basis of Cu accumulation and neurobehavioral impairments remains fragmentary. Previously, we have shown first *in vivo* evidence of spatial memory impairments along with astrocytes swelling (Alzheimer type II cells) and astrogliosis (increase in number of astrocytes), Cu deposition in the choroid plexus and degenerated neurons with significant increase in the hippocampus Cu content in Cu-intoxicated Wistar rats. In continuation with our previous study, the aim of this study was to investigate the effects of intraperitoneally injected Cu lactate (0.15 mg Cu/100 g body weight) daily for 90 days on metallothionein–I (MT-I), ATP7B, MT-III and acetylcholinesterase (AChE) gene expression by reverse transcription polymerase chain reaction. Cu-intoxicated group showed significantly increased expression of hepatic MT-I gene compared to control group. However, hepatic ATP7B mRNA levels in Cu-intoxicated group were comparable with that of control group. Similarly, MT-III and AChE gene expression in the brain were not significantly altered by chronic Cu-intoxication. In conclusion, the current study demonstrates that chronic Cu toxicity causes increase in hepatic MT-I mRNA levels in male Wistar rats.

Keywords: Copper intoxication; Metallothionein; Cognition; Neuro degeneration

Introduction

Copper ($_{29}Cu^{63.5}$) is the third-most abundant transition metal in the brain with highest concentration found in the liver. Owing to its redox activity, Cu serves as a cofactor for key metabolic enzymes that mediate various cellular processes, including neurotransmitter biosynthesis (dopamine β hydroxylase), mitochondrial energy generation (cytochrome *c* oxidase), free radical detoxification (Cu/Zn superoxide dismutase) and iron homeostasis (ceruloplasmin) [1,2]. There is uneven distribution of Cu in different regions of the brain with variations reported in different age groups [3], and species to species [4]. Average neural Cu concentrations are the order of 0.1 mM [1].

Brain is particularly sensitive to free radical damage and oxidative stress because of the high levels of polyunsaturated lipids that are component of neuronal cell membranes [5]. The abundance of Cu in the brain and its strong redox activity necessitates tight control over Cu homeostasis to evade oxidative injury to the central nervous system (CNS) by uncontrolled Cu-elicited Haber-Weiss and Fenton type reactions. Cells have therefore developed various protection mechanisms in event of drastic increase in Cu concentration. The incorporation of Cu into metallothionein (MT) is an example of cells defense mechanisms to protect cell structure from Cu toxicity and to prevent oxidative damage [6]. Four types of closely related MT genes (MT I-IV) are present in rodents [7,8]. While MT-I and MT-II are ubiquitously expressed, MT-III and MT-IV are primarily confined in the CNS and stratified squamous epithelia, respectively [9].

In recent years an overwhelming number of studies have shown relevance of MT in neurophysiological and neuromodulatory functions by documenting very high levels of brain-specific isoform MT-III in the CNS [10]. Haywood *et al.* have shown strong immunoreactivity for MT in the astrocytes of the North Ronaldsay sheep, an animal model for idiopathic Cu toxicosis (ICT) [11]. Increased expression of hepatic MT-I gene has been documented in toxic milk mice, an animal model of Wilson's disease (WD) [12]. It is well established that point mutation or a partial deletion in ATP7B gene, which encodes Cu transporting P-type ATPase, causes WD resulting in Cu accumulation in the liver, brain and cornea [13]. In addition, there is mounting proof that Cu dyshomeostasis in Alzheimer disease (AD) patients leads to oxidative stress and neuro degeneration [14] subsequently resulting in memory deficits. Central cholinergic system plays a significant role in learning, memory, and cognition in both animals and humans [15]. Notably, acetylcholinesterase (AChE) has been shown to be affected by Cu [16].

Earlier, we have reported a Wistar rat model for non-Wilsonian brain Cu toxicosis; documenting first *in vivo* evidence of memory deficits in conjunction with Cu deposition in the choroid plexus, decreased serum AChE activity, astrocytes swelling (Alzheimer type II cells) accompanied by astrogliosis (increase in number of astrocytes), degenerated neurons in cerebral cortex, and augmented levels of Cu in the hippocampus of chronically Cu-intoxicated male Wistar rats. In addition, liver sections of chronic Cu-intoxicated rats showed grade 1 Cu-associated protein and grade 4 Cu depositions by the Shikata's orcein stain and Rhodanine stain, respectively [17]. Importantly, MT has been confirmed as the biochemical counterpart of orcein positive material [18].

The widespread distribution of Cu prerequisite for normal CNS functioning, coupled with many links between Cu dyshomeostasis and neurodegenerative diseases, have impelled curiosity in studying MT, ATP7B and AChE gene expression in chronic Cu-intoxicated rats. In this study, we have reported the molecular basis of Cu accumulation in brain and liver with relation to development of neurodegeneration and neurobehavioral impairments in Wistar rat model for non-Wilsonian brain Cu toxicosis.

Materials and Methods

Chemicals

Copper chloride, agarose, chloroform, isopropyl alcohol (Merck) and lactic acid (Qualigens fine chemicals) were purchased. All the other reagents and chemicals used in this study were of analytical grade.

Animals and Experimental design

Male Wistar rats (starting at 3 weeks of age) in the weight range of 60–80 g were procured from the institute animal house, PGIMER, Chandigarh, India. All the rats were housed in the polypropylene cages (one animal per cage), kept in well ventilated rooms maintained at 22 ± 2 ˚C. The rats were fed standard rat chow and water ad libitum. Institutional Animal Ethical Committee (IAEC-161) consent was taken and IAEC guidelines were strictly followed for all the animal experimentation. Two groups of male Wistar rats, each consisting of eight animals were used as follows:

Control group: Intra peritoneal (i.p.) injection of isotonic sodium chloride solution daily for the period of 90 days. For ethical reasons, group receiving sodium lactate solution was not kept as it has been reported previously that it does not alter any vital biochemical parameters and Cu levels in various tissues in Wistar rats [17,19].

Cu-intoxication group: Intra peritoneal (i.p.) injection of Cu lactate solution (0.15 mg Cu/100 g. B.W.) daily for the period of 90 days [17].

Collection and preservation of tissue samples

All the animals were sacrificed at the end of 99th day of the study under ether anesthesia after completion of neurobehavioral studies [17], and brain and liver were dissected out. Tissues were cut with clean stainless steel scalpel blades. For expression studies, the liver and brain tissues were immediately transferred to autoclaved tubes containing Trizol® reagent. All the samples were stored at -80 ˚C till further processing.

Expression of MT-I, ATP7B, MT-III and AChE by semi-quantitative reverse transcription polymerase chain reaction (RT-PCR)

Total RNA was isolated from liver and brain tissues by Trizol® reagent (Invitrogen, USA). RNA purity was confirmed by 260/280 ratio. The expression of hepatic MT-I [20] and ATP7B gene [21] along with MT-III [20] and AChE [22] gene expression in brain tissues were determined by semi-quantitative RT-PCR using gene specific primers of MT-I, MT-III, ATP7B, AChE and β-actin as shown in table 1 with the following conditions: **for MT-I:** initial 2 min denaturation step at 94 ˚C, 45 sec at 94 ˚C, 1 min annealing at 58 ˚C, 72 ˚C for 1 min for 35 cycles; for **ATP7B:** initial 2 min denaturation step at 94 ˚C, 1 min at 94 ˚C, 1 min annealing step at 65 ˚C,1 min extension step at 72 ˚C for 35 cycles and a final 5 min extension at 72 ˚C; for **MT-III:** initial 2 min denaturation step at 94 ˚C, 1 min at 94 ˚C, 30 sec annealing at 52 ˚C, 1 min at 72 ˚C for 30 cycles; for **AChE:** initial 2 min denaturation step at 94 ˚C, 1 min at 94 ˚C, 1 min annealing step at 55 ˚C, 1 min extension step at 72 ˚C for 35 cycles and a final 7 min extension at 72 ˚C; for **β-actin:** initial 1 min denaturation step at 94 ˚C, 1 min at 94 ˚C, 1 min annealing step at 58.5 ˚C, 1 min extension step at 72 ˚C for 35 cycles and a final 10 min extension at 72 ˚C. cDNA were prepared by using a RevertAid first strand cDNA synthesis kit (Fermentas) according to manufacturer's protocol. To ensure that equal amounts of reverse transcribed RNA were added to PCR reaction, a parallel amplification of β-actin mRNA was performed as an internal reference [22]. Gel pictures were taken in Alpha Imager®. Signal intensities of the MT-I, ATP7B, MT-III and AChE products were normalized to those of β-actin products as ratios to produce arbitrary units of relative abundance. Band intensities of the RT-PCR products were quantified by densitometry using Image densitometry software.

Statistical analysis

All values were expressed as the mean ± standard error of the mean (SEM) of eight animals in each group. Unpaired Student's t test and Mann–Whitney rank sum test were used for analysis of the data, and values with $p < 0.05$ were considered statistically significant. All the calculations were carried out by the Sigma Stat computer software program.

S.no	Gene	Primer sequence	Product Size (bp)
1	Acetylcholinesterase (AChE)	F-5'-GAC TGC CTT TAT CTT AAT GTG-3' R-5'-CGG CTG ATG AGA GAT TCA TTG-3'	785
2	MT-I	F-5'-CCCACTGCTCCTGCTCCAC-3' R-5'-GTCACTTCAGGCACAGCACG-3'	185
3	MT-III	F-5'-ATGCAGCTGCTGCCAGTGAG-3' R-5'-TGTGCATGGGATTTATTCAC-3'	151
4	ATP7B	F5'CCTCGAGAATTACATCACCGACACTG3' R5'CCCACCACAGCCAGAACCTTCCTGA3'	332
5	β-actin	F-5'TAT GCC AAC ACA GTG CTG TCT GG-3' R-5'-TAC TCC TGC TTC CTG ATC CAC AT-3'.	288

Table 1: Primer sequences of ATP7B, MT-I, MT-III, AChE and β-actin genes for expression studies using semi-quantitative RT-PCR

Results

Effects of chronic Cu-intoxication on hepatic MT-I and ATP7B gene expression

Total RNA obtained from liver tissue was used for making cDNA, and then PCR was performed for hepatic MT-I with respective primers as given in Table 1. Hepatic MT-I mRNA fold change in Cu-intoxicated group in comparison to control group are depicted in Figure 1. Semiquantitative study of MT-I mRNA using RT-PCR reactions revealed increased expression of MT-I gene in liver tissues of Cu-intoxicated group as compared to control group (Figure 1). However, there were no significant changes in the expressions levels of hepatic ATP7B gene in Cu-intoxicated group (Data not given).

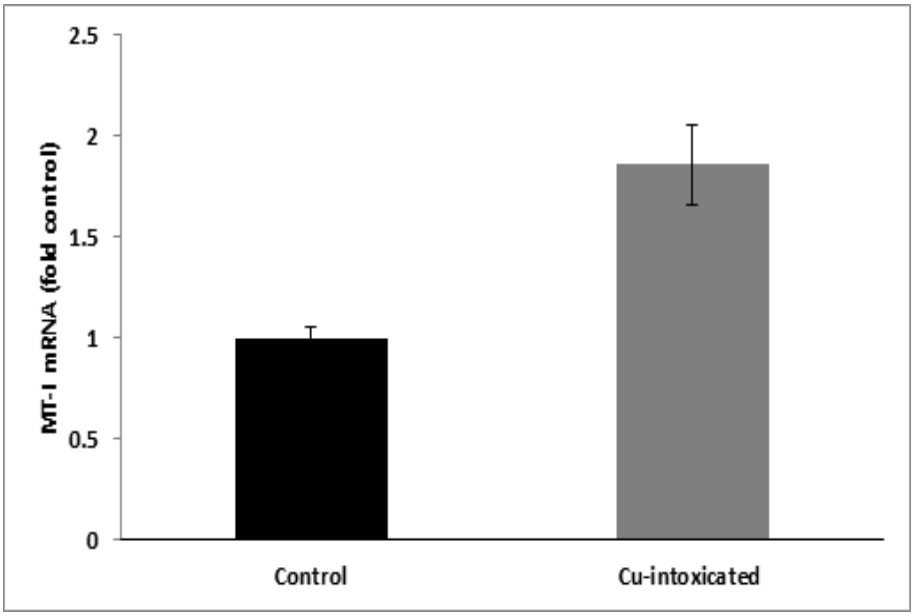

Figure 1: Histogram showing the fold changes in the mRNA expression of MT-I gene in liver tissues Cu-intoxicated group in comparison to control group

Effects of chronic Cu-intoxication on MT-III and AChE gene expression in the brain

Total RNA extracted from brain tissue was used for making cDNA, and then PCR was performed for AChE and MT-III genes separately with respective primers as given in Table 1. Expressions levels of MT-III, which is a brain specific isoform of MT, in brain tissues of Cu-intoxicated showed non-significant changes compared to control group (Data not given). Similarly, AChE gene mRNA levels in Cu-intoxicated group were comparable with that of control group (Data not given).

Discussion

The present study documents molecular basis of chronic Cu-intoxication elicited hepatic and neurotoxicity with increased expression of hepatic MT-I gene (Figure 1) in Wistar rat model for non-Wilsonian brain Cu toxicosis [17]. However, there were no changes in the mRNA levels of hepatic ATP7B due to chronic Cu-intoxication. Similarly, no significant effect of Cu-intoxication was found on the MT-III and AChE gene expression in the brain. Results described here point towards the major role of hepatic MT-I in liver Cu accumulation due to chronic Cu-intoxication corroborated by increased hepatic MT-I gene expression (Figure 1). In addition, we had also shown grade 1Cu-associated protein by Shikata's orcein stain and massive grade 4 Cu deposition by rhodanine stain, respectively in liver sections of Cu-intoxicated animals in the histopathological studies [17]. Liver sections of co-

trol animals were negatively stained with rhodanine and Shikata's orcein stain. Evans *et al.* have established that MT is the constituent of the orcein positive material [18]. Thus, implying that MT-I levels are up regulated at both transcription (mRNA) and translation (protein) levels which is substantiated by MT-I RT-PCR (Figure 1) and histopathological studies with orcein stain, respectively. MT-I mRNA levels were also found to be increased in the toxic milk mice [12] which is parallel with our observations. With increased hepatic Cu loading, MT immunoreactivity was found to be increased periportally with robust panlobular staining for MT in North Ronaldsay sheep, which displays an uncharacteristic sensitivity to dietary Cu. Further, Simpson *et al.* have also revealed that hepatic Zn concentrations did not increase with increased MT immunoreactivity, thereby, confirming that MT expression was consistent with increase in hepatic Cu [23], which concurs well with decreased hepatic Zn content and Cu-associated protein in our previously reported non-Wilsonian rat model of brain Cu toxicosis [17].

However, there were no significant changes on the MT-III gene expression in the brain of Cu-intoxicated animals in our study. The orcein staining also failed to detect Cu-associated protein in histopathological studies of the brain of Cu-intoxicated animals [17]. Haywood *et al.* [11] have shown increased immunoreactivity for MT in astrocytes of North Ronaldsay sheep (brain Cu content 343.5 μg Cu/g). MTs are ubiquitous proteins characterized by high cysteine and metal content. It is well established that the expression of MT-I proteins is vastly inducible in response to a variety of stimuli, including metals, oxidative agents, inflammation, hormones, cytokines, and stress [24,25]. MTs are induced by toxic metal ions such as cadmium (Cd), mercury and cobalt or essential trace elements such as Zn and Cu. In addition, MTs also binds to Cd, Zn and Cu [26,27]. At the cellular level, MT is mainly distributed in the cytoplasm and to a lesser extent in the nuclei and lysosomes [28,29]. An overexpression of MT can be caused by Zn or Cu exposure and this induction may be due to the role of MTs as antioxidants and electrophilic scavengers [30,31]. Regulation of MT biosynthesis by metals has been considered as a biological device to maintain essential and non-essential free metal ions homeostasis by their chelation. Metal-induced synthesis is mediated through the action of short cis-acting DNA sequences known as metal responsive elements (MREs), which are present in the promoter region of all mammalian MT genes [32,33]. Nevertheless, the regulation of MT-III gene expression is poorly known [9].

The mechanisms for Cu accumulation due to chronic Cu-intoxication seems apparently different from those observed in WD as evident by no changes in the mRNA levels of the hepatic ATP7B gene, and increased serum ceruloplasmin and serum Cu levels in Cu-intoxicated animals [17]. Copper Metabolism gene MURR1 containing Domain 1 (COMMD1), is a recently identified gene which has been hypothesized as essential for Cu excretion at the biliary pole of hepatocytes, acting downstream ATP7B. Its mutation is responsible for Bedlington terrier copper toxicosis (BTCT), an animal model of WD [34]. ATP7B is found primarily localized in hepatocytes and it is vital for the regulation of Cu homeostasis in mammals [35]. When extracellular Cu concentration is below 1 μmol/L, hepatic ATP7B is located in the trans-Golgi network (TGN) and delivers Cu ions to vital cuproenzymes; however, under augmented Cu stress ATP7B sense the increased Cu levels and translocate to the biliary canaliculi and to vesicular structures to excrete the surplus of intracellular Cu [36-38]. The function of ATP7B in tissues other than liver is unclear [37].

Non-Wilsonian forms of liver Cu toxicosis in humans that often occur early in childhood include endemic Tyrolean infantile cirrhosis (ETIC) [39], Indian childhood cirrhosis (ICC) [40], and ICT [41]. Genetic defects in these rare diseases of Cu toxicosis have not been identified until now, but high dietary Cu ingestion or consumption of inorganic Cu in drinking water and supplements [42], and consanguinity are reported to be involved in the pathogenesis of these Cu toxicosis diseases, which clearly indicates towards genetic cause altered by environmental and dietary/drinking water factors [34].

AChE hydrolyses cholinergic neurotransmitter, acetylcholine, to acetate and choline. Acetylcholine transmits the nerve impulse from one neuron to another i.e. from the presynaptic terminus, through the synaptic cleft to the specific receptors on postsynaptic terminus, so that propagation of the signal can take place. Acetylcholine is involved in memory, learning, control of motor tone, locomotion, cortical activation, attention, pain and control of autonomic functions [43]. The cholinergic system is involved in synaptic plasticity; memory and learning with deficits in it are reported to be robustly related to neurodegenerative diseases [44]. In our study, significant decrease in the serum AChE activity [17] along with non-significant changes on the AChE gene expression were documented in Cu-intoxicated animals. In concurrence, Cu toxicity has been shown to induce neurobehavioral effects probably by affecting the "cholinergic anti-inflammatory pathway" [45]. Nevertheless, molecular mechanism for decrease in AChE activity due to chronic Cu-intoxication is not known. Leiva et al. [46] have suggested a possible relationship between Cu and cholinergic receptors. Actually, Farrar and Hoss [47] have shown that brain areas with a high concentration of Cu, such as hippocampus, cerebral cortex, forebrain and striatum, overlap sites of cholinergic innervation [46]. Cholinergic agonists can facilitate memory, whereas cholinergic antagonists can impair memory [15,48]. From the above quoted references, we can say that Cu toxicity can diminish the AChE enzyme activity without altering the AChE gene expression, and the observed impaired AChE activity could interfere with cholinergic neurotransmission, affecting learning, memory, cognition and motor functions of Cu-intoxicated animals.

Conclusion

In conclusion, in the present study it was found that hepatic MT-I gene expression increased significantly but hepatic ATP7B gene, and brain MT-III and AChE gene expression remained unaltered due to chronic Cu-intoxication in Wistar rat model for non-Wilsonian brain Cu toxicosis. However, more studies addressing the molecular mechanisms underlying neuronal apoptosis due

to buildup of Cu in different regions of brain together with interaction between different cells of brain parenchyma (neurons, astrocytes, microglia, oligodendroglia and endothelial cells) in the CNS Cu homeostasis are warranted to elucidate molecular mechanisms of chronic Cu toxicity associated cognitive deficits and neurodegeneration.

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