Toxicology and Applied Pharmacology 237 (2009) 205–213

Contents lists available at ScienceDirect

Toxicology and Applied Pharmacology

journal homepage: www.elsevier.com/locate/ytaap

Copper accumulation and compartmentalization in mouse fibroblast lacking metallothionein and copper chaperone, Atox1

Takamitsu Miyayama [a,b], Kazuo T. Suzuki [a], Yasumitsu Ogra [a,b,*]

[a] Graduate School of Pharmaceutical Sciences, Chiba University, Chuo, Chiba 260-8675, Japan
[b] Laboratory of Chemical Toxicology and Environmental Health, Showa Pharmaceutical University, Machida, Tokyo 194-8543, Japan

ARTICLE INFO

Article history:
Received 20 February 2009
Revised 18 March 2009
Accepted 29 March 2009
Available online 9 April 2009

Keywords:
Atox1
Copper
CS1
Ctr1
Metallothionein

ABSTRACT

Copper (Cu) is the active center of some enzymes because of its redox-active property, although that property could have harmful effects. Because of this, cells have strict regulation/detoxification systems for this metal. In this study, multi-disciplinary approaches, such as speciation and elemental imaging of Cu, were applied to reveal the detoxification mechanisms for Cu in cells bearing a defect in Cu-regulating genes. Although Cu concentration in metallothionein (MT)-knockout cells was increased by the knockdown of the Cu chaperone, Atox1, the concentrations of the Cu influx pump, Ctr1, and another Cu chaperone, Ccs, were paradoxically increased; namely, the cells responded to the Cu deficiency despite the fact that cellular Cu concentration was actually increased. Cu imaging showed that the elevated Cu was compartmentalized in cytoplasmic vesicles. Together, the results point to the novel roles of MT and cytoplasmic vesicles in the detoxification of Cu in mammalian cells.

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Introduction

Copper (Cu) is an essential trace element in living organisms. It functions as the active center of cuproenzymes, such as cytochrome *c* oxidase (CCO), Cu,Zn-superoxide dismutase (Cu,Zn-SOD, SOD1), ceruloplasmin, lysyl oxidase, tyrosinase, and dopamine β-hydroxylase (Massaro, 2003). The mechanisms underlying Cu homeostasis are suggested as follows (Kim et al., 2008); Cu is mainly incorporated into cells as a monovalent Cu ion, i.e., cuprous ion, by Ctr1, a transporter expressed on the plasma membrane (Lee et al., 2000; Zhou and Giyschier, 1997). Incorporated Cu associates with one of three cytoplasmic Cu escort proteins, the so-called Cu chaperones, for it to be escorted to specific organelles or cuproenzymes. Atox1, a Cu chaperone for the Golgi apparatus, hands over Cu to Atp7a and Atp7b, which are ATP-dependent Cu transporters into the secretory pathway of Cu via the Golgi apparatus (Klomp et al., 1997; Hamza et al., 1999). In the Golgi apparatus, Cu is incorporated into cuproenzymes, such as ceruloplasmin and lysyl oxidase, due to the secretion of these cuproenzymes into extracellular fluid. Otherwise, Cu is directly excreted from cells by secretory vesicles that translocate to the plasma membrane to exocytose Cu. Cox17, a Cu chaperone for the mitochondria, is required to donate Cu to CCO and/or SCO1, which is a recipient protein of Cu on the mitochondrial membrane (Horng et al., 2005; Horng et al., 2004). CCS, a Cu chaperone for SOD1, transports Cu to SOD1 in cytosol by forming a heterodimer with SOD1. In addition to these Cu chaperones, a novel Cu-regulating protein, Commd1, was recently characterized (Maine and Burnstein, 2007). It does not have apparent Cu-binding motifs in its molecule but is proposed to participate in the Cu-efflux pathway by interacting with Atp7b (de Bie et al., 2005). Indeed, Bedlington terriers demonstrated inherited hepatic Cu toxicosis due to a defect in Commd1 (Forman et al., 2005). Metallothionein (MT) is suggested to be also a Cu-regulating protein. It actually binds Cu via Cu-thiolate clusters (Presta and Stillman, 1997). Since MT binding of Cu is thermodynamically and kinetically stable, excess Cu is sequestered by MT to mask Cu toxicity (Salgado and Stillman, 2004). On the other hand, an alleviative role of MT in Cu deficiency was also suggested (Ogra et al., 2006; Suzuki et al., 2002). Thus, MT may play a dual role in Cu homeostasis in mammalian cells.

As mentioned above, Cu is regulated by various proteins, such as transporters across the membrane, chaperones in the cytoplasm, and MT that buffers Cu availability in cells. However, this rigid regulation may prevent us from experimentally disturbing intracellular Cu concentration and further analyzing the mechanisms of Cu regulation. In particular, MT is induced by either an excess or a deficiency of Cu to maintain Cu homeostasis. In some cases, it was suggested that MT did not contribute to the detoxification of Cu. For instance, it is reported that Formosan squirrels abnormally and inheritably accumulated Cu in the liver (Suzuki et al., 2004). Although other inherited Cu toxicosis animals, such as toxic milk mice and Long–Evans Cinnamon (LEC) rats, accumulated Cu in the form bound to MT in their livers (Shim and Harris, 2003), it was shown that Cu accumulated in the liver of Formosan squirrels dominantly existed in the insoluble fraction of the liver and only a small amount was bound to MT (Suzuki et al., 2004). Alternatively, Cu that was rapidly administered to rats also existed in

* Corresponding author. Laboratory of Chemical Toxicology and Environmental Health, Showa Pharmaceutical University, Machida, Tokyo 194-8543, Japan. Fax: +81 42 721 1563.
E-mail address: ogra@ac.shoyaku.ac.jp (Y. Ogra).

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doi:10.1016/j.taap.2009.03.024

the form that was not bound to MT (Suzuki et al., 1989). However, these animals did not manifest Cu toxicity and thus, a detoxification mechanism that functions independently of MT is speculated. In this study, we intended to establish an experimental model in which Cu concentration was modified by gene knockdown (KD) in a Cu-regulating protein, and to show that the contribution of MT to Cu homeostasis could be ignored. In this regard, fibroblasts established from an MT-null mutant mouse, i.e., MT-knockout cells (MT-KO) cells, are frail against the Cu toxicity, and those cells are more useful to reveal Cu homeostasis than MT-wild type (MT-WT) cells. Hence, Atox1-KD was introduced into MT-KO cells.

In the present study, multi-disciplinary approaches were used to reveal the detoxification mechanisms for Cu in cells. First, speciation was used to determine the chemical species of Cu in the soluble fraction of cells. As the sample from cultured cells was too small to allow for analysis of Cu species by conventional HPLC coupled with an inductively coupled plasma mass spectrometer (ICP-MS), micro HPLC coupled with ICP-MS was adopted. Second, an elemental fluorescent probe for Cu, Cu sensor 1 (CS1), was used for Cu imaging. This probe enables visualization of intracellular Cu distribution in live cells. Thus, elemental imaging is a complementary technique to elemental speciation in metallochemical biology. The aim of this study is to reveal the MT-independent detoxification mechanisms for Cu using MT-KO cells bearing Atox1-KD with multi-disciplinary approaches.

Materials and methods

Reagents. Milli-Q water, 18.3 MΩ/cm, (Millipore) was used throughout. Tris(hydroxymethyl)aminomethan (Trizma®Base) was purchased from Sigma (St. Louis, MO, USA). Ammonium acetate, acetic acid, 28% ammonia solution, hydrochloric acid, and other reagents of the highest grade were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

Cell culture and gene knockdown with siRNAs. MT-wild type (MT-WT) and MT-knockout (MT-KO) cells were established from embryonic fibroblasts of 129Sv MT-WT and MT-KO mice transformed with SV40 large T antigen by Kondo et al. (Kondo et al., 1999) and kindly provided by Professor Seiichiro Himeno (Tokushima Bunri University, Japan). Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) of the high glucose type (4500 mg/l), and supplemented with 10% heat inactivated fetal bovine serum, 10 U/ml penicillin, and 100 μg/ml streptomycin at 37 °C under 5% CO_2 atmosphere. Double-stranded RNAs (dsRNAs) were used as siRNAs (Stealth RNAi, Invitrogen, Tokyo, Japan). The targeted sequence of Atox1 was as follows: siRNA_Atox1, 5′-ACAAGCUGGGAGGAGUGGA-GUUCAA-3′. MT-WT and MT-KO cells were seeded on a six-well plastic plate at 1.0×10^5 cells/well, and were pre-incubated for 24 h. The pre-incubated cells were either transfected or not transfected with 100 nM

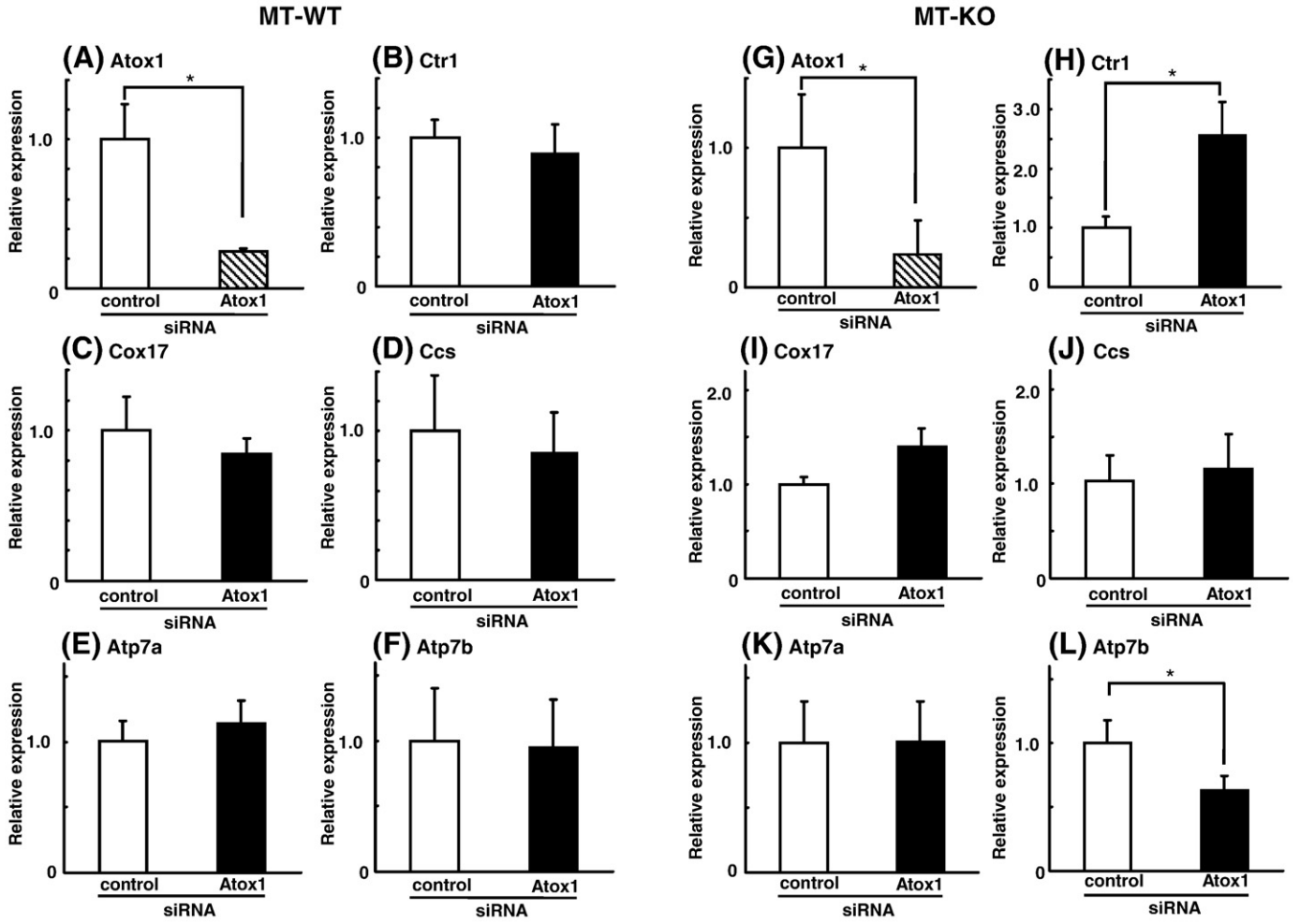

Fig. 1. Effects of Atox1-KD on the mRNA expression of Cu-regulating genes in MT-WT and MT-KO cells. MT-WT (A–F) and MT-KO (G–L) cells were pre-incubated for 24 h and the cells were treated with control or Atox1 siRNA at 100 nM for 24 h. After the siRNA transfection, total RNA was isolated from the cells. The mRNA expressions of Atox1 (A and G) and other Cu-regulating genes, such as Ctr1 (B and H), Cox17 (C and I), and Ccs (D and J), were quantified by RT-PCR analysis and normalized to β-actin levels. Atox1 expression was significantly knocked down by the siRNA treatment in MT-WT and MT-KO cells (A and G). Ctr1 mRNA expression was significantly increased by Atox1-KD in MT-KO cells (H). Data are expressed as means ± SD of three independent determinations.

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