Title
Low concentrations of copper in drinking water increase AP-1 binding in the brain

Permalink
https://escholarship.org/uc/item/83j7n9wq

Journal
Toxicology and Industrial Health, 31(12)

ISSN
0748-2337

Authors
Lung, S
Li, H
Bondy, SC
et al.

Publication Date
2015

DOI
10.1177/0748233713491805

Peer reviewed
Low concentrations of copper in drinking water increase AP-1 binding in the brain

Shyang Lung, Huihui Li, Stephen C. Bondy and Arezoo Campbell

Toxicol Ind Health published online 29 May 2013
DOI: 10.1177/0748233713491805

The online version of this article can be found at:
http://tih.sagepub.com/content/early/2013/05/29/0748233713491805

Published by:
SAGE
http://www.sagepub.com

Additional services and information for Toxicology and Industrial Health can be found at:

Email Alerts: http://tih.sagepub.com/cgi/alerts
Subscriptions: http://tih.sagepub.com/subscriptions
Reprints: http://www.sagepub.com/journalsReprints.nav
Permissions: http://www.sagepub.com/journalsPermissions.nav

>> OnlineFirst Version of Record - May 29, 2013

What is This?
Low concentrations of copper in drinking water increase AP-1 binding in the brain

Shyang Lung1, Huihui Li2, Stephen C. Bondy2 and Arezoo Campbell1

Abstract
Copper (Cu) in trace amounts is essential for biological organisms. However, dysregulation of the redox-active metal has been implicated in different neurological disorders such as Wilson’s, Menkes’, Alzheimer’s, and Parkinson’s diseases. Since many households use Cu tubing in the plumbing system, and corrosion causes the metal to leach into the drinking water, there may be adverse effects on the central nervous system connected with low-level chronic exposure. The present study demonstrates that treatment with a biologically relevant concentration of Cu for 3 months significantly increases activation of the redox-modulated transcription factor AP-1 in mouse brains. This was independent of an upstream kinase indicated in AP-1 activation. Another redox-active transcription factor, NF-κB, was not significantly modified by the Cu exposure. These results indicate that the effect of Cu on AP-1 is unique and may involve direct modulation of DNA binding.

Keywords
AP-1, copper, NF-κB, neurodegenerative diseases, oxidative stress

Introduction
Copper (Cu) is a cofactor necessary for the function of many enzymes required in living organisms. The importance of Cu homeostasis in brain function is demonstrated by two hereditary disorders caused by mutations in genes encoding P-type cation-transporting adenosine triphosphatases. These genetic abnormalities lead to disturbances in the absorption and distribution of the metal. These disorders include Menkes’ disease, which results in neurological degeneration, and Wilson’s disease, which leads to liver toxicity and neurological impairments (Kitzberger et al. 2005; Strecker et al., 2006; Vulpe et al., 1993). Cu levels are also altered in age-related neurodegenerative disorders such as Alzheimer’s disease (AD) and Parkinson’s disease (PD). The non-ceruloplasmin-bound component of the metal is increased in the serum of patients with AD (Squitti et al., 2005) and the cerebrospinal fluid of patients with PD (Hozumi et al., 2011). One of the characteristic hallmarks of AD is senile plaques composed of amyloid fibrils derived from the amyloid precursor protein (APP). It has been demonstrated that interaction of Cu with the Cu regulatory element motif in the promoter region regulates gene expression of APP (Bellingham et al., 2004). Therefore, an increase in Cu concentration may contribute to APP overexpression and amyloid deposition. Furthermore, prolonged occupational exposure to Cu is linked to an increased risk of PD (Gorell et al., 1999).

Many households use Cu tubing in the plumbing system and there is concern regarding the potential adverse effects on the central nervous system (CNS) connected with chronic low-level Cu exposure (Marx, 2003). The present Environmental Protection Agency’s maximum recommended standard for Cu in drinking water is 1.3 ppm (Buchanan et al., 1999). However, the levels can be as high as 7.8 mg/L in tap water that has been stagnant overnight in corrosive Cu pipes (Spitalny

1 Department of Pharmaceutical Sciences, College of Pharmacy, Western University of Health Sciences, Pomona, CA, USA
2 Center for Occupational and Environmental Health, University of California, Irvine, Irvine, CA, USA

Corresponding author:
Arezoo Campbell, Department of Pharmaceutical Sciences, College of Pharmacy, Western University of Health Sciences, 309 E Second Street, Pomona, CA 91766-1854, USA.
Email: acampbell@westernu.edu
et al., 1984) and as high as 5 mg/L in running tap water (Pizarro et al., 2001). In the present study, the effect of a 3-month exposure to Cu (0.5 ppm) in the drinking water upon the activator protein-1 (AP-1) and nuclear factor-κB (NF-κB) activation was investigated. Activation of both these transcription factors has been reported to underlie apoptotic cell death in striatal neurons (Qin et al., 2001).

Methods

Animal treatment

Two-month-old male B/6C3F1 mice (a hybrid between C57BL/6 and C3H) were obtained from Charles River Laboratories (Wilmington, Massachusetts, USA). They were housed four per cage and maintained on a 12-h light/dark cycle in a temperature-controlled (20 ± 1°C) room. Food and water were provided ad libitum. The drinking water was supplemented with 8 μM of copper sulfate. This is equivalent to 0.5 ppm of Cu. Control mice received double distilled water. The amount of water consumed was recorded twice a week, and the weight of the animals was determined each week. The animals were exposed for 3 months. For brain tissue collection, animals were anesthetized with pentobarbital (65 mg/kg, intraperitoneally). Depth of anesthesia was ascertained by the toe pinch reflex. When animals were deeply sedated, the chest cavity was opened and mice were perfused transcardially with phosphate-buffered saline to remove blood from all organs. The brain was removed and quickly frozen.

Preparation of samples

Cytoplasmic and nuclear fractions were prepared using a previously published method (Lahiri and Ge, 2000). The brain tissue from each animal was weighed and homogenized in 2 mL of an ice-cold buffer (10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), pH 7.9, 10 mM potassium chloride (KCl), 0.1 mM ethylenediaminetetraacetic acid (EDTA), 0.1 mM ethylene glycol tetraacetic acid (EGTA), 1 mM dithiothreitol (DTT), 0.5 mM phenylmethylsulfonyl fluoride (PMSF), and 0.5 mM phenylmethylsulfonyl fluoride (PMSF). The samples were then centrifuged at 11,000g for 5 min at 4°C. The supernatant (nuclear extract) was aliquoted and stored at −80°C.

Electrophoretic mobility shift assay

The extent of AP-1 and NF-κB activation was determined in the nuclear fraction of brain tissue using a protocol developed by Promega Corporation (Madison, Wisconsin, USA). The amount of protein in 5 μL of the nuclear extract was determined by the bicinchoninic acid (BCA) protein assay method (Pierce, Rockford, Illinois, USA) and 50 μg of each sample, incubated with 32P-labeled oligonucleotides containing either the AP-1 or NF-κB consensus sequence, was loaded onto a gel. A negative control containing no cell extract, as well as competitor reactions were included. The specific competitor contained unlabelled AP-1 or NF-κB consensus nucleotide, while the nonspecific competitor contained unlabelled SP-1 consensus oligonucleotide. The competitor reactions also contained 50 μg of nuclear fraction derived from Cu-treated mouse brains. X-Ray films were manually developed, and the intensity of each band was measured and quantitated using an image analyzer.

Western blots

The levels of interleukin-1 receptor-associated kinase 1 (IRAK-1), NF-κB-inducing kinase (NIK), c-Jun N-terminal kinase (JNK), and phospho-JNK (p-JNK) were determined using Western blotting. Protein content was determined using the BCA protein assay kit. Each sample of 50 μg was resolved on a sodium dodecylsulfate–10% polyacrylamide gel electrophoresis and transferred onto a nitrocellulose membrane (BioRad, Hercules, California, USA). After blocking overnight in Tris-buffered saline and Tween 20 (TBST; 20 mM Tris-HCl, 150 mM NaCl, and 0.1% Tween 20)-containing 5% nonfat milk, membranes were washed four times and incubated for 1 h with primary antibodies (goat polyclonal antibody against p-JNK (1:500), rabbit polyclonal antibody against JNK (1:500), mouse monoclonal antibody against IRAK-1 (1:250), and rabbit polyclonal antibody against NIK (1:500); all purchased from Santa Cruz Biotechnology, Inc., Santa Cruz, California, USA). After washing the membranes four times with TBST, they were incubated with appropriate secondary horseradish peroxidase-conjugated antibodies (1:10,000). Bands were detected with enhanced-chemiluminescence reagents (Amersham...
Pharmacia Biotech, Piscataway, New Jersey, USA) following the manufacturer’s protocol. An antibody against actin (mouse monoclonal, 1:1500 dilution) purchased from Chemicon International (Temecula, California; USA) was used to insure equal loading of protein for each sample. The intensity of specific bands was measured and quantitated using an image analyzer.

Statistical analysis
The differences among control and Cu exposure were assessed by the Student’s t test using Prism software (GraphPad Software, San Diego, California, USA). Results were considered statistically significant at p < 0.05 using a two-tailed distribution.

Results
A 3-month exposure to copper sulfate (8 μM), in the drinking water, did not cause any significant changes in the weight or water consumption of treated animals when compared with the controls (data not shown). This implied that the overall health of animals was not affected by treatment. There was a significant increase in the levels of AP-1 DNA binding in Cu-exposed mouse brains compared with the controls as judged by gel shift mobility assay (Figure 1). Since the phosphorylation of JNK is an upstream event, which precedes the activation of AP-1, the levels of the active form of JNK, that is p-JNK, and JNK were assessed. These were unchanged after exposure (Figure 2). Therefore, Cu exposure may directly affect DNA binding without activating upstream signaling events. The activation of another transcription factor, NF-κB, was also assessed (Figure 3(a)). Integrated density measurement of the main shifted band revealed that Cu exposure did not significantly alter the levels of NF-κB DNA binding in the nuclear fraction of mouse brain (Figure 3(b)). The levels of two kinases related to NF-κB activation were evaluated. The brain levels of the NIK were not changed. A reduction in IRAK-1 was evident but not statistically significant (Figure 4). Prolonged exposure to low levels of Cu in the drinking water modulates AP-1 DNA binding but upstream signaling pathways do not appear to be responsible for this effect.

Discussion
In the present study, the levels of AP-1 DNA binding were increased in the CNS of mice chronically exposed to biologically relevant concentrations of Cu. This is in agreement with a previous study showing that a 4- or 24-h exposure to high concentrations of Cu (400 μM) increased AP-1 binding in COS-7 cells (Mattie et al., 2008). Since this transcription factor is modulated by the redox status of cells (Mattson et al., 2004), such activation may be in response to an increase in prooxidant events. Indeed, chronic exposure to Cu significantly increased the lipid peroxidation marker, malondialdehyde, and neuronal nitric oxide synthase levels in the brain (Becaria et al., 2006).

The enhancement of AP-1 DNA binding after a 3-month exposure to Cu was not associated with a concurrent increase in the phosphorylation of JNK, which activates the signaling pathway for this transcription pathway. It is possible that during an acute response, the levels of this mitogen-activated protein kinase increased but that the longer duration of exposure allows homeostasis to be reached. This is in agreement with the in vitro findings of Mattie et al. (2008), where a 4-h exposure caused a significant increase in JNK phosphorylation that was attenuated by a 24-h exposure. The effect of Cu on transcription factor activation may also be mediated by direct

![Figure 1. AP-1 activation. (a) Typical gel shift mobility assay showing three separate samples per group (B: blank; C: control; Cu: copper; SC: specific competitor containing sample with unlabelled AP-1 consensus oligonucleotide; NSC: nonspecific competitor containing sample with unlabelled SP-1 consensus oligonucleotide). (b) Integrated density of the main shifted band. Bars represent mean of six individual animal determinations ± SE. *Value is significantly different (p ≤ 0.05) from the control.](image-url)
modulation of the affinity of active AP-1 for its DNA-binding sites. The activation of both AP-1 and NF-κB has been shown to enhance apoptosis in striatal neurons (Nakai et al., 2000). Intrastriatal injection of dopamine, which can also act as an oxidant, induced apoptotic changes that were dependent on AP-1 and NF-κB activation (Luo et al., 1999). Therefore, it may be that Cu increased the DNA binding of AP-1 by way of its prooxidant properties. This could be a mechanism by which the metal acts as a neurotoxicant and contributes to neurological impairments.

In recent years, the potential role of Cu in the pathology of AD has received much attention. Concentrations of the metal are increased in amyloid plaques, where it strongly colocalizes with the β-sheet conformation of aggregated amyloid proteins (Miller et al., 2006). There are specific Cu-binding sites on both the APP (Barnham et al., 2003) and amyloid beta (Aβ) peptide (Dong et al., 2003), and they increase the toxicity of the metal by reducing Cu(II) to Cu(I). This allows redox cycling, with consequent formation of reactive oxygen species within CNS tissue (Dikalov et al., 2004; Feaga et al., 2011; Guilloreau et al., 2007). In the present study, we show that Cu increases AP-1 DNA binding. Since AP-1 is modulated by the redox status of cells, the mechanism of action of Cu is more likely to initially relate to the promotion of oxidative processes (Becaria et al., 2006). This is in agreement with the work of Mattie et al. (2008), which showed that increasing oxidative stress caused enhanced AP-1 binding in COS-7 cells.

Trace amounts of Cu (0.12 ppm), present in the drinking water, increase the number of Aβ reactive neurons and senile plaque-like structures in a rabbit model of AD (Sparks and Schreurs, 2003). The promoter region of the β-site APP-cleaving enzyme contains multiple transcription factor binding sites, including ones for AP-1 and cyclic adenosine monophosphate response element-binding protein (Lahiri et al., 2006). Cu-induced increase in the activation of AP-1 may thus contribute to accelerated formation of Aβ. This may be a potential mechanism by which the metal enhances the number of Aβ reactive neurons (Sparks et al., 2006). This effect of metals on potentiation of AD pathology may not be unique to Cu. In monkeys, exposure to lead (Pb), during brain development,
led to an increase in APP expression and amyloid deposition (Wu et al., 2008). Under SP-1 transcription regulation, fetal exposure to Pb caused latent promotion of APP expression later in life (Basha et al., 2005). The “Latent Early-life Associated Regulation” model explains how exposure to environmental agents (such as Cu) may cause changes in oxidation status of promoters, which modify latent expression of disease-associated genes (Lahiri et al., 2009).

Early gene imprinting by environmental factors such as metal exposure, and subsequent altered responses of AD-associated genes, may be an important etiological pathway for neurodegenerative disorders (Lahiri and Maloney, 2010).

Free Cu cation has been correlated with cognitive decline (Salustri et al., 2010) and this may affect the initiation of the prodrome of AD (Brewer, 2012; Campbell, 2006). It has been suggested that failure of Cu sequestration by microglia clustered around amyloid plaques may precipitate inflammatory conditions and thus exacerbate AD (Zheng et al., 2010). Further studies are needed to determine the exact mechanism by which Cu enhances DNA binding of AP-1 and determine whether exposure to this metal at environmentally relevant levels may accelerate the progression of neurodegenerative diseases such as AD.

**Funding**

This study was supported by grants from Western University of Health Sciences (12085-1501) and the National Institutes of Health (ES 7992).

**References**


