Eur Biophys J (2008) 37:315–321
DOI 10.1007/s00249-007-0244-1

REVIEW

The role of metals in modulating metalloprotease activity in the AD brain

Gulay Filiz · Katherine A. Price · Aphrodite Caragounis · Tai Du · Peter J. Crouch · Anthony R. White

Received: 17 August 2007 / Revised: 18 November 2007 / Accepted: 20 November 2007 / Published online: 13 February 2008

Abstract Biometals such as copper and zinc have an important role in Alzheimer's disease (AD). Accumulating evidence indicates that copper homeostasis is altered in AD brain with elevated extracellular and low intracellular copper levels. Studies in animals and cell cultures have suggested that increasing intracellular copper can ameliorate AD-like pathology including amyloid deposition and tau phosphorylation. Modulating copper homeostasis can also improve cognitive function in animal models of AD. Treatments are now being developed that may result in redistribution of copper within the brain. Metal ligands such as clioquinol (CQ), DP-109 or pyrrolidine dithiocarbamate (PDTC) have shown promising results in animal models of AD, however, the actual mode of action in vivo has not been fully determined. We previously reported that CQ-metal complexes were able to increase intracellular copper levels in vitro. This resulted in stimulation of phosphoinositol-3-kinase activity and mitogen activated protein kinases (MAPK). Increased kinase activity resulted in up-regulated matrix metalloprotease (MMP2 and MMP3) activity resulting in enhanced degradation of secreted Aβ. These findings are consistent with previous studies reporting metal-mediated activation of MAPKs and MMPs. How this activation occurs is unknown but evidence suggests that copper may be able to activate membrane receptors such as the epidermal growth factor receptor (EGFR) and result in downstream activation of MAPK pathways. This has been supported by studies showing metal-mediated activation of EGFR through ligand-independent processes in a number of cell-types. Our initial studies reveal that copper complexes can in fact activate EGFR. However, further studies are necessary to determine if metal complexes such as CQ-copper induce up-regulation of Aβ-degrading MMP activity through this mechanism. Elucidation of this pathway may have important implications for the development of metal ligand based therapeutics for treatment of AD and other neurodegenerative disorders.

Keywords Alzheimer's disease · Metal complex · Metaligand based therapeutics · Metaloproteins · Epidermal growth factor receptor · Neurodegenerative disorders

Australian Society for Biophysics Special Issue: Metals and Membranes in Neuroscience, held in Melbourne on 11 July 2007.

G. Filiz · K. A. Price · A. Caragounis · T. Du · P. J. Crouch · A. R. White (✉)
Department of Pathology and the Centre for Neuroscience, The University of Melbourne, Melbourne, VIC 3010, Australia
e-mail: arwhite@unimelb.edu.au

G. Filiz · K. A. Price · A. Caragounis · T. Du · P. J. Crouch · A. R. White
The Mental Health Research Institute, Parkville, VIC 3052, Australia

Introduction

Alzheimer's disease (AD) is a progressive neurodegenerative disorder characterized by extracellular accumulation of amyloid beta (Aβ) peptide, intracellular formation of neurofibrillary tangles (NFTs) and synaptic dysfunction (Glenner and Wong 1984; Masters et al. 1985). Currently, no effective treatment exists for AD. A growing body of evidence now supports a central role for altered biometal metabolism in development and progression of AD (Bush 2003). In particular, the transition metal, copper, has been implicated in Aβ aggregation and neurotoxicity as well as altered processing of the amyloid precursor protein (APP) (Barnham et al. 2004b; White et al. 2006b). These findings

suggest that amelioration of AD pathology may be achieved through modulation of copper homeostasis.

Copper homeostasis and AD

Copper homeostasis in AD is complex and the mechanisms of copper trafficking, metabolism and sequestration are currently being elucidated. One of the key findings emerging from these studies is that there is an excess of copper in the extracellular space in the brain (Maynard et al. 2005; Crouch et al. 2006). Studies by Lovell et al. (Lovell et al. 1998) demonstrated that the AD neuropil contained copper levels approximately 4.4-fold higher than in control brains. Copper levels were elevated even further in amyloid plaques. This accumulation of extracellular copper is likely to be mediated largely by copper binding to the Aβ peptide. Aβ has both high (K_{app} 10) and low (K_{app} 7) affinity copper binding sites (Atwood et al. 2000; Cuajungco et al. 2000). Bush and colleagues have published extensively on the interaction between copper and Aβ. They have shown that low levels of copper i.e., 1 μM, can induce substantial aggregation of synthetic peptide (Maynard et al. 2005). In addition, copper binding to Aβ can result in production of hydrogen peroxide through reduction of copper(II) to copper(I) (Behl et al. 1994; Huang et al. 1999). Subsequent interaction between hydrogen peroxide and copper(I) can then result in generation of highly toxic hydroxyl radical species (Barnham et al. 2004a). Recent evidence suggests that low molecular weight oligomers of Aβ induced by copper-binding can insert into cell membranes or bind to specific receptors and may induce hydroxyl radical mediated oxidative stress and neuronal dysfunction (Barnham et al. 2003; White et al. 2006a).

However, in contrast to increased extracellular copper levels, intracellular copper appears to be diminished in AD brain compared to controls. Deibel et al. (1996) reported that overall copper levels (intra- and extra-cellular) in hippocampus were decreased by approximately 20% in AD compared to control brain. This is despite the increase in extracellular copper (Lovell et al. 1998). Moreover, the activity of several cuproenzymes is diminished in AD, including copper/zinc superoxide, cytochrome c oxidase (COX) and peptidylglycine alpha amidating monooxygenase (Duara et al. 1986; Wand et al. 1987; Maurer et al. 2000; Cottrell et al. 2001). This is despite an apparent increase in protein levels for some of these enzymes (Omar et al. 1999; Bayer et al. 2003).

Modulation of copper homeostasis controls Aβ metabolism

Although the mechanisms involved are yet to be determined, intracellular copper levels appear to control Aβ levels. This has been supported by evidence showing that increasing intracellular copper results in reduction of secreted Aβ levels. Borchardt et al. (1999) demonstrated that in cells over-expressing APP, exposure to high copper resulted in a decrease in secreted Aβ. More recently, Phinney et al. (2003) and Bayer et al. (2003) reported that increasing central nervous system copper levels in brains of AD transgenic mice by genetic or dietary means resulted in decreased Aβ deposition in the brain. These studies have provided the basis for the hypothesis that efficient delivery of copper to the brain could ameliorate one of the central pathological hallmarks of AD, amyloid deposition. Alternatively, agents that can re-distribute copper from the extracellular space to the intracellular environment to restore copper homeostasis also have a strong potential as AD therapeutics.

Evidence is now accumulating to support these hypotheses. Cherny et al. (2001) reported that AD transgenic (Tg2576) mice treated with the lipid soluble metal complexing agent, clioquinol (CQ) revealed elevated CNS copper and decreased amyloid deposition. This has subsequently been reported for another lipid soluble metal ligand, DP-109 (Lee et al. 2004). More recently, Malm et al. (2007) described the treatment of AD transgenic mice (APP/PS1) with pyrrolidine dithiocarbamate (PDTC), a well-known copper complexing agent. In this study, a 20% increase in CNS copper was observed in treated mice together with improved cognitive function, although no obvious changes to Aβ deposition were reported. We have observed similar effects with alternate metal ligands (unpublished observations). These phenomena may be explained by changes to discrete Aβ oligomers (toxic species?) or localized changes to Aβ resulting in improved cognitive function but without overall effect on Aβ deposition.

Despite the success of metal complexing agents at ameliorating AD-like pathology in animal models, it is still uncertain how these agents induce their protective effects in vivo. Previous studies suggested that CQ can interfere with metal binding to extracellular Aβ, resulting in inhibition of Aβ aggregation and enhanced Aβ turnover (Cherny et al. 1999, 2001; Huang et al. 1999, 2004). This was supported by in vitro (synthetic Aβ) and ex vivo (AD brain tissue) studies showing dissolution of Aβ-copper by CQ. However, whether this occurs in vivo has not been fully addressed.

Copper complexes induce degradation of Aβ by metalloproteases in vitro

To investigate alternate mechanisms of metal ligand action, we examined the effects of CQ on Aβ levels in APP-transfected cells. We found that CQ was able to substantially

increase the cellular copper levels when cultures were treated with CQ-copper complexes (White et al. 2006b; Caragounis et al. 2007). The increase in cellular copper correlated with a dramatic and rapid decrease in levels of extracellular Aβ including both Aβ1-40 and Aβ1-42. Similar effects were observed in neuronal cells. Subsequently, we investigated the pathways involved and found that CQ-copper complexes triggered stimulation of phosphoinositol-3-kinase (PI3K) resulting in downstream phosphorylation of Akt and inhibition of glycogen synthase kinase 3β (GSK3β) (White et al. 2006b; Caragounis et al. 2007). This latter effect potentiated JNK activation and together with ERK phosphorylation, resulted in increased synthesis of matrix metalloprotease 2 and 3 (MMP2 and MMP3). Up-regulation of MMP2 and MMP3 activity resulted in potent cleavage of extracellular Aβ, thus preventing accumulation of Aβ in conditioned medium (White et al. 2006b; Caragounis et al. 2007). These findings suggested that modulation of MMP activity by metals could affect Aβ turnover in AD.

Metalloproteases and AD

It has been reported that several different metalloproteases can degrade Aβ. However, the role of metals in modulating expression or activation of these proteases has not been extensively studied (Carson and Turner 2002). Angiotensin-converting enzyme (ACE), insulin degrading enzyme (IDE), neprilysin (NEP) and MMPs all have established Aβ cleavage activity in vitro and/or in vivo (Carson and Turner 2002). Little is known about the effects of metals on the activity of these enzymes other than the active site zinc. The only study on the role of Cu in ACE activity is by Reeves et al. (1990) who demonstrated that ACE activity was increased by 20–30% in kidneys of rats fed a Cu-deficient diet, however, the activity of brain ACE was not determined and the mechanism by which lower Cu levels increases ACE activity is not known.

Studies have found that NEP is also associated with Aβ degradation (Eckman and Eckman 2005). NEP-knockout mice revealed reduced degradation of exogenously administered Aβ1-42 compared to wild-type controls (Eckman and Eckman 2005). Furthermore, the endogenous levels of Aβ1-40 and Aβ1-42 were considerably increased in the brains of the NEP-knockout mice, suggesting that NEP is a physiologically important Aβ-degrading metalloprotease (Eckman and Eckman 2005). Recent reports demonstrate that NEP expression and activity is modulated by various factors that are associated with AD (Eckman and Eckman 2005). Aging is the major risk factor for the development of AD (Eckman and Eckman 2005). Therefore, it has been proposed that the heightened risk of AD with increasing age may be due to the down-regulation of NEP and other Aβ-degrading metalloproteases. Several studies have shown that NEP is susceptible to oxidative damage by metal-mediated oxidation and that this can increase susceptiblility to proteolysis (Fisk et al. 2007). NEP may however, be more resistant than other Aβ-degrading metalloproteases to oxidative damage (Fisk et al. 2007).

MMPs are a well characterized family of zinc-metalloproteases that are responsible for modulating the interaction between cells and surrounding matrix. This is important for cell growth, differentiation, synaptic plasticity, angiogenesis and many other processes. MMPs can also cleave ligands from cell surfaces and therefore have an important role in cell-cell signalling. In addition, abnormal MMP activity is associated with cancer and arthritis. MMPs are enzymes that have been shown to cleave Aβ in vitro (Backstrom et al. 1996). MMPs can be released from neurons, microglia, astrocytes, oligodendrocytes, leukocytes and endothelial cells, and their target molecules include collagen, gelatin, fibronectin and proteoglycans as well as other components of the cell matrix (Oh et al. 1999; Harkness et al. 2000). MMP expression and activity is regulated at many levels including the transcriptional level, where MMP expression is modulated by cytokines, free radicals and growth factors (Yong et al. 1998; Beuche et al. 2000). MMP activity depends on the activation of latent proforms and endogenous tissue inhibitors of metalloproteases (TIMPs) (Brew et al. 2000). The TIMP family is comprised of four related proteins (TIMP-1,-2,-3 and -4) that are able to bind MMPs to form tight, non-covalent inhibitory complexes within the catalytic site of MMPs resulting in the inhibition of MMP activity. MMP expression has been investigated in AD and other neurodegenerative disorders, and some studies claim that levels of MMPs and TIMPs are increased while others claim that levels are decreased or have no change (Adair et al. 2004).

MMPs and Aβ

It has been shown that these MMPs are capable of degrading Aβ. MMP-2 cleaves at residues Lys^{16}-Leu^{17}, Leu^{34}-Met^{35} and Met^{35}-Val^{36}, MMP-3 cleaves at Glu^{3}-Phe^{4} (Rapala-Kozik et al. 1998; Asahi et al. 2001) and MMP-9 cleaves at Aβ positions Lys^{16}-Leu^{17}, Ala^{30}-Ile^{31}, Leu^{34}-Met^{35} and Gly^{37}-Gly^{38} (Roher et al. 1994; Rapala-Kozik et al. 1998). MMP-9 is localised to neurons of the hippocampus, and its expression is heightened in senile plaques, neurofibrillary tangles and in the vascular wall in postmortem AD brain tissue (Asahi et al. 2001). Studies on AD plasma have found increases in MMP-9 expression, and it has been suggested that the elevated levels (Lorenzl et al. 2003) may be due to endothelial cells releasing MMP-9 in response to circulating Aβ and homocysteine. Other groups

have suggested that MMPs are synthesised in response to Aβ, and if the latent form of MMP is activated it would further degrade the Aβ peptide in vivo and reduce the aggregation of the peptide in the plaques (Backstrom et al. 1996).

Previous studies have reported that both MMP2 and MMP3 can be up-regulated by copper, although excess free metal can also inhibit MMP activity (Adamson et al. 2003; Wu et al. 2004). Activation of MMP2 and MMP3 can occur through the stimulation of PI3K and MAPK pathways (Kubiatowski et al. 2001; Matsumoto et al. 2004) although the triggering mechanism for this is not known. Accumulating data suggests that metal-mediated activation of membrane receptors could be responsible for activation of signalling pathways leading to increased MMP activity. We have shown recently that a number of different metal ligand complexes including 8-hydroxyquinoline and phenanthroline derivatives can induce activation of MMPs and decrease extracellular Aβ (Caragounis et al. 2007) (Fig. 1). Importantly, our studies showed that some of these agents could induce substantial loss of Aβ with little or no measurable increase in cellular metal levels. Paradoxically, the effects were only induced by ligands with a high-lipid solubility. Our interpretation is that metal complexes may be interacting with membranes or specific membrane domains (i.e., lipid rafts) and induce activation of membrane-bound

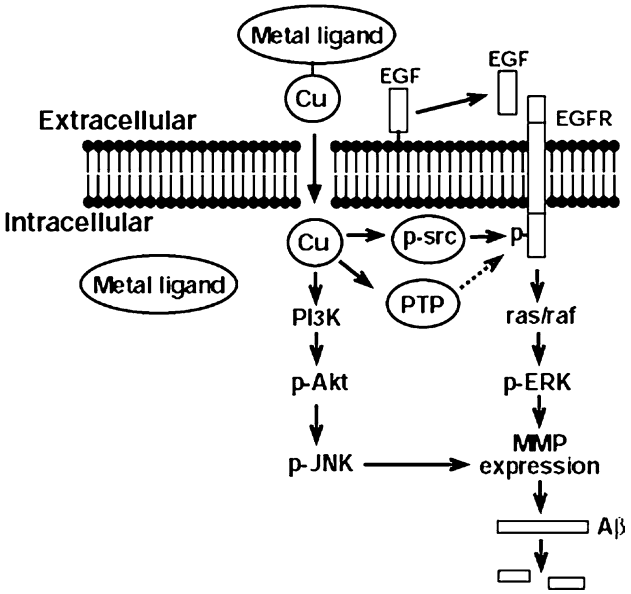

Fig. 1 Schematic of proposed pathway for modulation of matrix metalloprotease expression by metal-complexes. The lipid permeability of the metal ligand results in increased intracellular Cu levels. The Cu induces activation of src kinase (p-src) and inhibition of protein tyrosine phosphatases (*PTP*) leading to phosphorylation of EGFR. Cu may also induce release of cognate EGFR ligands such as EGF or HB-EGF. Activation of EGFR induces up-regulation of ERK activity (p-ERK) and through a synergistic effect with PI3K-JNK pathway, increases metalloprotease expression. This results in increased cleavage of the Aβ peptide

receptors. Support for this comes from the extensive body of work published by Wu et al. (2004) and Samet et al. (2003). This group has found that exposure of various cell-types to high concentrations of metals especially zinc but also copper, results in activation of the epidermal growth factor receptor (EGFR).

EGFR

EGFR is a 170 kDa membrane-spanning protein widely expressed in mammalian cells (Samet et al. 2003). Its intracellular kinase domain is activated upon ligand binding by EGF (Yarden 2001; Samet et al. 2003). EGF binding to the extracellular domain promotes the formation of a receptor homodimer (Earp et al. 1995), which is autophosphorylated on its tyrosine residues (Schlessinger and Ullrich 1992). This acts as a high affinity binding site for molecules downstream of the receptor that contain the SH2 src homology domain (Schlessinger 1994; Pawson 1995). Autophosphorylation of EGFR occurs at ~5 tyrosine sites, the main ones are tyrosines 1068, 1173 and 1148, the minor sites are tyrosine residues 992 and 1086 (Hackel et al. 1999). Phosphorylation of tyrosine 845 on EGFR can also occur. Cognate ligand binding to EGFR can induce the activation of downstream pathways including the PI3K-Akt pathway (Wu et al. 2003), and the ras/raf-MAP kinase pathway, which subsequently directs cells to proliferate, differentiate and/or survive (Cameron et al. 1998; Schlessinger 2002; Wong 2003). This is controlled via the modulation of further downstream molecules such as GSK3 and ERK, which in turn affect a range of cellular activities including cytoskeletal re-arrangement, process outgrowth and regulation of cell adhesion by matrix metalloproteases (MMPs). As a result of being able to produce these effects in cells, a possible role for EGFR in neurodegenerative diseases is emerging. Research suggests that activation of EGFR can stimulate the survival and growth of rat cortical neurons in low-density cultures, and may have a role in astrocyte development, neuronal migration- and synaptic-plasticity in hippocampal neurons (Abe and Saito 1992). Furthermore, it was reported that aged brains can respond to exogenous growth factors (EGF) with increased neurogenesis. This implies that there is therapeutic potential in the use of growth factors, neurotrophic factors, or compounds that mimic their activity in the treatment of age-related neurodegenerative diseases such as AD.

EGFR and activation by metals

Normal activation of the EGF receptor involves binding of EGF or several other known ligands to the extracellular

domain of the receptor, but emerging studies indicate that it can also be stimulated by metals such as zinc and copper (Wu et al. 2004). Samet et al. reported zinc-induced phosphorylation of tyrosines 1068, 845 and 1173 on EGFR that was independent of receptor dimerization and intracellular kinase activation (Samet et al. 2003). It was found that zinc-induced EGFR activation resulted from trans-activation of the receptor via the non-receptor tyrosine kinase c-Src, which can cause phosphorylation of certain tyrosine residues without receptor dimerization (Samet et al. 2003). This was supported by the observation that pre-treatment of cells with the c-Src inhibitor PP2 abolished zinc dependent phosphorylation at Tyr845 and Tyr1068 (Samet et al. 2003).

Additional studies by this group suggest that EGFR activation by metals may also occur through an autocrine mechanism in cells. Wu et al. found that the EGF ligand, HB-EGF was released from Zn^{2+} treated human bronchial epithelial cells, thereby inducing phosphorylation of EGFR (Wu et al. 2004). This autocrine mechanism of HB-EGF on EGFR was supported when it was shown that treatment with an antibody against the extracellular ligand-binding domain of EGFR was able to block Zn^{2+}-induced receptor activation by HB-EGF [129]. Released HB-EGF was also detected in cultured cell media following treatment with Zn^{2+} (Wu et al. 2004). These findings suggest that metal-driven activation of EGFR may occur via more than one mechanism, including direct interaction with the receptor, activation of upstream signalling molecules (c-Src), or may cause release of ligand from cells which can then act on EGFR's extracellular ligand binding domain.

EGFR activation results in stimulation of downstream pathways including PI3K and MAPK, the same pathways activated by metal complexes and resulting in increased MMP activity in our cell cultures (Fig. 1). Whether EGFR or alternative membrane receptors are activated by metal complexes and stimulates MMP synthesis is not known. We are currently investigating these mechanisms and have shown that EGFR is phosphorylated by CQ-Cu in cell culture and that inhibition of EGFR activation modulates the levels of secreted Aβ peptide (unpublished observations). Further studies on these pathways will potentially identify whether these mechanisms are involved and may lead to novel drug targets for AD and other neurodegenerative disorders.

Copper partitioning for dual therapeutic benefit in AD

As we have shown in this discussion, there are two critical aspects of Cu homeostasis to be addressed in relation to potential AD therapies. Increased levels of extracellular Cu appears to contribute to Aβ aggregation and subsequent neurotoxicity through localized free radical generation. Therefore, one of the obvious therapeutic targets is to reduce extracellular Cu in the brain or prevent the metal from interacting with Aβ. This was the aim of the initial CQ treatment of transgenic AD mice by Cherny et al. (2001). The idea was to interfere with Cu binding to Aβ by using a low affinity Cu ligand (CQ). As reported previously (Cherny et al. 2001; White et al. 2006a), the evidence supports the ability of CQ to act in this manner. However, recent studies by White et al. (2006b) and Caragounis et al. (2007) suggest that metal ligands of the 8-hydroxyquinoline family, including CQ, may be able to modulate Aβ production by acting as Cu or Zn ionophores. The metal potentially bound by CQ or other 8-hydroxyquinolines in the extracellular space (including metal removed from extracellular Aβ) may be delivered into the neighbouring cells due to the cell permeable nature of the ligands. As reported previously (Wu et al. 2004; White et al. 2006b; Caragounis et al. 2007) and discussed here, this may trigger signalling EGFR activation and downstream modulation of MMP activity leading to Aβ degradation. This proposed dual activity of metal ligands could help to reduce levels of neurotoxic Aβ through partitioning of brain Cu between the intra and extracellular environment.

Summary

In conclusion, metal ligands are currently being investigated as potential therapeutic agents for treatment of AD (Cherny et al. 2001; Ritchie et al. 2003). The in vivo action of these agents is yet to be fully determined but increasing evidence suggests that modulation of metal homeostasis may be an important effect. Altering cellular metal levels with metal complexes can activate cellular signalling pathways leading to up-regulation of Aβ degrading MMP activity. The effects of metal ligands and complexes on other Aβ degrading metalloproteases needs to be investigated, Similarly, whether altered expression or activation of metalloproteases occurs through metal-mediated membrane receptor activation requires further investigation.

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