# 13.10 Protein Phosphatase 1 as a Potential Mediator of Aluminum Neurotoxicity

**E F da Cruz e Silva**, Universidade de Aveiro, Aveiro, Portugal

**J P O'Callaghan**, Centers for Disease Control and Prevention – NIOSH, Morgantown, WV, USA





## Abbreviations

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|---|---|
| **AD** | Alzheimer's disease |
| **A$\beta$** | Abeta |
| **CAMP** | 3′-5′ cyclic adenosine monophosphate |
| **GABA** | gamma-aminobutyric acid |
| **$IP_3$** | inositol 1,4,5-trisphosphate |
| **MAPK** | mitogen-activated protein kinase |
| **PI-3** | phosphoinositide-3 |
| **PP1** | protein phosphatase 1 |
| **PP2A** | protein phosphatase 2A |
| **PP2B** | protein phosphatase 2B |
| **PTEN** | phosphatase and tensin homologue |

## 13.10.1 Introduction

Reversible protein phosphorylation is a ubiquitous and universal process generally regarded as the major post-translational modification through which numerous physiological processes are regulated (Cohen 2002a; de la Fuente van Bentem *et al.* 2008). It is a dynamic process whereby phosphate groups are incorporated into proteins by protein kinases and removed by protein phosphatases. Thus, protein phosphorylation is a reversible process that alters the function and/or localization of target proteins and in this fashion controls various biological functions. In turn, the protein kinases and protein phosphatases, which are particularly abundant and diverse in mammalian brain, are themselves regulated by a myriad of extracellular and intracellular signals. Many of the complex functions of the mammalian central nervous system are regulated by phosphorylation/dephosphorylation of key regulatory proteins. Many extracellular messengers exert their effects in the central nervous system by modulating the intracellular concentration of specific second messengers, which in turn lead to the activation/inhibition of specific protein kinases and phosphatases. As a result, abnormal phosphorylation of key regulatory proteins has been associated with a variety of disease conditions and dysfunctional states. Defects in protein phosphorylation mechanisms operating in the mammalian central nervous system may be a common feature of many neurological and chemical-induced disease states (Gong and Iqbal 2008; Lee and Messing 2008).

## 13.10.2 Protein Phosphatases

Until recently the protein kinases were regarded as the key regulatory component of tripartite protein phosphorylation systems (a substrate protein, a phosphorylating kinase, and a dephosphorylating phosphatase). However, the exquisite complexity of diverse metabolic processes regulated by protein phosphorylation cannot be explained solely by kinase regulation. Ultimately, the level of phosphorylation of a particular substrate protein depends on the fine equilibrium between the relevant kinase and phosphatase activities. Available data indicate that it is the interaction of phosphatase catalytic subunits with regulatory and targeting subunits that dictate their cellular location

and control catalytic activity and substrate specificity (Moorhead *et al.* 2009; Virshup and Shenolikar 2009). Recent focus on protein phosphatases not only raised awareness of their importance in neuronal systems, but also led to the realization that phosphatases are often the targets of neurotoxins. Several natural toxins and toxicants of medical and environmental importance have been identified as potent and specific inhibitors of the serine/threonine-specific protein phosphatases (Nishiwaki-Matsushima *et al.* 1992; Suganuma *et al.* 1988).

### 13.10.3 Altered Protein Phosphorylation and Neurotoxicity

The correlation of specific neurotoxic conditions to changes in protein phosphorylation is relatively scarce. Most efforts have been directed toward neurological disorders such as Alzheimer's disease (AD), and little effort has been devoted to the role of protein phosphorylation in neurotoxicity induced by metals like aluminum (da Cruz e Silva and O'Callaghan 1997). Abnormal hyperphosphorylation of tau and other paired helical filament proteins seems to be a central component of AD pathology and it was suggested that phosphatases may play an overlooked role (Iqbal and Grundke-Iqbal 1998). However, the known sites of action of neurotoxins and neurotoxicants are as diverse and varied as the cell types and processes that compose the central nervous system. The central and fundamental role of protein phosphorylation systems in neuronal function implies that the action of an environmental toxicant will likely lead to alterations in protein phosphorylation (O'Callaghan 1994; da Cruz e Silva and O'Callaghan 1997). Since many of the sites of action of neurotoxicants, for example, neurotransmitter receptors and transporters, are known substrates for phosphatases, the role of the latter in neurotoxicity remains largely neglected.

One might assume that a lack of current attention to potential roles for phosphatases in neurotoxic responses might speak to the lack of evidence for the role of these enzymes in any aspect of neural injury. Such is not the case, however, as there is ample evidence for the involvement of phosphatases in neuropathology in both the peripheral and the central nervous systems. Indeed, phosphatase-related neuropathology is known to exist at multiple levels. Among the many lines of evidence supporting this contention are the (1) effects of loss/mutation of specific phosphatases, (2) participation of phosphatases as downstream effectors in oxidative stress-related neuronal damage, and (3) disrupted neural physiology and brain function due to irreversible blockade of dephosphorylation of specific phosphoprotein substrates. Thus, the mutation of myotubularin-related gene encoding a phosphatase acting on phosphoinositide-3 (PI-3) results in a demyelinating peripheral neuropathy (Previtali *et al.* 2003). In addition, mutated phosphatase and tensin homologue (PTEN), another phosphatase regulating signal transduction through PI-3 kinase, results in a highly selective lesion of the cerebellum restricted to the granular layer (Abel *et al.* 2005). In contrast to these genetic loss-of-function examples, inhibition of the MAPK phosphatases (primarily PP2A) activity in response to sustained upstream activation of MAPK during oxidative stress contributes to neuronal death (Ho *et al.* 2008). These findings raise the possibility that oxidative imbalance and an accumulation of reactive oxygen species can contribute to neuronal damage via inhibition of phosphatases. Therefore, inhibition of selected phosphatases, leading to oxidative stress, may underlie a variety of neurological diseases and neurotoxic outcomes where oxidative stress is implicated. Finally, abrogated phosphatase actions may be involved in conditions where specific phosphoserines are organophosphorylated by a variety of nerve agents, rendering these sites irreversibly blocked to the normal action of phosphatases (O'Callaghan 2003). This may result not only in acute neurotoxicity and death, but also in more subtle but prolonged adverse outcomes. For example, low-level exposure to the nerve agent sarin results in persistent learning and memory deficits due to alterations in key signal transduction pathways yet to be fully elucidated (Henderson *et al.* 2002). In aggregate these examples attest to the multiple levels and targets involved in neurotoxic events leading to adverse pathological outcomes that have been shown to involve phosphoprotein phosphatases. Given the myriad of phosphorylation events that underlie normal neural physiology, and that can be regulated by phosphatases as well as kinases, it is a certainty that phosphatases serve as targets, modulators, and mediators of diverse neurotoxic events.

One of the most significant advances in the study of the serine/threonine-specific protein phosphatases was the discovery that several naturally occurring toxins (e.g., okadaic acid and microcystin) exert their effects by specifically inhibiting protein phosphatases (Bialojan and Takai 1988; MacKintosh *et al.* 1990). Although structural studies have revealed the presence of metal ions in the phosphatase catalytic sites and indicate that metal ions are required for

phosphatase activity (Goldberg *et al.* 1995; Griffith *et al.* 1995), relatively few studies have addressed the effect of metals on protein phosphatases. Here, we will focus on the neurotoxic actions of aluminum and the possibility that these can be explained via direct inhibition of a specific type of protein phosphatase that is particularly enriched in the mammalian brain – protein phosphatase 1 (PP1). Aluminum exposure can result in altered protein phosphorylation and is associated with many pathological features related to AD. Many studies have demonstrated the ability of aluminum to induce neuronal cytoskeletal abnormalities consistent with altered neurofilament phosphorylation (Shea and Fischer 1991), but the molecular mechanisms responsible for the observed aluminum-induced hyperphosphorylation need to be further elucidated. Nonetheless, it has been suggested that aluminum-induced inhibition of phosphatase activity may explain the observed neuropathological effects of the metal (Shetty *et al.* 1992). As discussed below, more recent data indicate that aluminum does indeed inhibit PP1 activity directly and may, therefore, lead to hyperphosphorylation of physiological PP1 substrates. Of course, the exact effects of aluminum depend on the nature of the aluminum compound, the route and duration of exposure, and the animal species involved.

## 13.10.4 Human Intake and Bioavailability of Aluminum

Aluminum is the third most abundant element in nature, exhibiting high bioavailability even though there is no known biological process that requires it. Daily human exposure results mainly from food consumption, with drinking water contributing only around 3% of total daily aluminum intake. Personal hygiene products, such as antiperspirants and toothpaste, also contain considerable concentrations of aluminum that consequently can be absorbed through the skin (Exley 2004). Other potential sources of human exposure include occupational (McLachlan 1995) and dental prostheses and vaccines where it is used as an adjuvant (Brewer 2006). Although the absorption rate of aluminum is relatively low (Moore *et al.* 2000), human exposure is virtually unavoidable and occurs during the entire life span. However, in this context it is interesting to note that aluminum absorption may increase with aging. Taylor *et al.* (1992) reported that young individuals absorb much less aluminum from an aluminum citrate drink than older individuals.

Once in the bloodstream, aluminum binds to plasma proteins (e.g., transferrin and albumin), complexes with citrate (Fatemi *et al.* 1991), and is distributed throughout the body. Although aluminum accumulates mainly in the skeleton, it is also found in liver, kidney, muscle, and heart (Kerr *et al.* 1992; Walker *et al.* 1994). Additionally, it is generally accepted that the most plausible mode of entry of aluminum into the brain is through the blood–brain barrier (Yokel 2002). Aluminum itself affects the permeability of the blood–brain barrier, enhancing its lipophilicity and contributing to an increase in transmembrane diffusion (Meiri *et al.* 1993). Once in the brain, aluminum competes with elements such as Fe, Ca, or Mg present in various proteins and enzymes, potentially altering their function.

## 13.10.5 The Effect of Aluminum in the Nervous System

The molecular mechanisms underlying aluminum-dependent neurotoxicity remain unclear, although a wide range of neuronal effects have been associated with aluminum exposure. Aluminum affects behavior, neurotransmission, energy metabolism, and various signal transduction pathways in a variety of mammalian species. Aluminum-induced intraneuronal neurofilamentous aggregates characterized by argentophilic masses in neuronal perikarya, proximal axonal enlargements, and proximal dendrites have been reported (Gotow 2000; Gotow *et al* 1995; Troncoso *et al.* 1986). Those aggregates were suggested to comprise abnormally phosphorylated neurofilament proteins and that their phosphorylation state determined neurofilament structural organization. Abnormally phosphorylated tau protein has also been observed in neurofilament aggregates induced by aluminum (Huang *et al.* 1997; Singer *et al.* 1997). Indeed, the direct injection of aluminum into the central nervous system of rabbits mimics neuropathological and biochemical abnormalities characteristic of AD and related human neurodegenerative disorders (Huang *et al.* 1997). Intracisternal injection of aluminum was also reported to produce many of the clinical, histological, and ultrastructural alterations associated with amyotrophic lateral sclerosis, including argentophilic perikaryal inclusions and neurofibrillary tangle-like structures (Wakayama *et al.* 1996). Many human neurodegenerative diseases have been associated with abnormal phosphorylation of cytoskeletal proteins and consequent neurofilamentous aggregates. It is

proposed that the predominance of negative charges in such hyperphosphorylated proteins would tend to make such protein aggregates unstable. Thus, a positively charged species, such as aluminum, might promote and stabilize neurofibrillary aggregates, as observed in AD and in aluminum-induced neurofibrillary degeneration (Savory *et al.* 2001).

Behavioral abnormalities such as spatial disorientation, decreased activity, increased emotionality, cognitive and motor function deficits, and changes in learning and memory have all been reported in animals exposed to aluminum (Julka *et al.* 1995; Kaneko *et al.* 2006; Miu *et al.* 2003; Oteiza *et al.* 1993; Roig *et al.* 2006). Aluminum impairment of hippocampal long-term potentiation, a model for synaptic plasticity underlying some forms of learning and memory, was reported both *in vivo* and *in vitro* (Platt *et al.* 1995). More recently it was suggested that enhancement of inflammation and interference with cholinergic projections might explain the learning and memory deficits caused by aluminum (Platt *et al.* 2001). In fact, aluminum also affects other neurotransmitters, namely glutamatergic, GABAergic, serotonergic, and dopaminergic systems (El-Rahman 2003; Kumar 2002; Milanese *et al.* 2001; Nayak and Chatterjee 2001; Tsunoda and Sharma 1999; Yang *et al.* 2003).

A reduction in glucose metabolism was observed in rat brain following chronic aluminum exposure (Clauberg *et al.* 1994), but more recently it was shown that aluminum enhanced the activity of glucose-6-phosphate dehydrogenase (Kaur and Gill 2006). However, aluminum was previously reported to inhibit the activities of both hexokinase and glucose-6-phosphate dehydrogenase (Cho and Joshi 1989; Exley *et al.* 1994). In conclusion, aluminum probably interferes with many biological processes due to its high reactivity.

Among the signal transduction pathways that have been reported to be disrupted by aluminum are inositol 1,4,5-trisphosphate ($IP_3$), cAMP-mediated signaling, and intracellular calcium homeostasis. Initial observations indicated that aluminum inhibits receptor-mediated $IP_3$ production in neuroblastoma cells (Shi *et al.* 1993). Competitive inhibition of phospholipase C by aluminum was observed in various brain regions (Nostrandt *et al.* 1996).

## 13.10.6 Aluminum Inhibition of PP1

PP1, one of the major types of serine/threonine-specific protein phosphatases, is ubiquitously distributed and has been implicated in such diverse biological processes as synaptic plasticity, dopaminergic neurotransmission, cell cycle progression, muscle contraction, and glycogen metabolism (Bollen 2001; Cohen 2002b; Virshup and Shenolikar 2009). In mammals three genes encode type 1 phosphatase catalytic subunits, termed PP1$\alpha$, PP1$\beta$ (also known as PP1$\delta$), and PP1$\gamma$, which share >90% homology between themselves (da Cruz e Silva *et al.* 1995). Neurofilament L (Terry-Lorenzo *et al.* 2000) and tau protein (Liao *et al.* 1998) are among the many proteins known to interact directly with PP1. Tau probably targets PP1 to microtubules, with PP1 being involved in the maintenance of microtubule stability. Further, PP1 is also involved in the dephosphorylation of tau, although other phosphatases such as PP2A and PP2B may be more relevant in this context (Gong *et al.* 2005). At the protein level PP1$\alpha$ and PP1$\gamma$ are more highly expressed in brain than in peripheral tissues, with the highest levels being observed in the basal ganglia, where both are particularly enriched in the medium-sized spiny neurons (da Cruz e Silva *et al.* 1995). At the electron microscopic level, PP1 immunoreactivity is highly and specifically enriched in dendritic spine heads and necks (Ouimet *et al.* 1995), a strategic location for regulation of signal transduction cascades.

Our recent work indicates that aluminum exposure results in decreased expression and diminished activity of PP1 (Amador *et al.* 2004), a concentration and time-dependent effect that was consistently observed in different cell lines. For example, incubation of PC12 cells with 0.5 mmol $l^{-1}$ $AlCl_3$ for 24 h yielded an inhibition of PP1 activity of around 25%. Additionally, experimental $IC_{50}$ values were calculated using purified recombinant PP1$\alpha$ and PP1$\gamma_1$ as 0.28 and 0.26 mmol $l^{-1}$, respectively. Although the precise molecular link between aluminum neurotoxicity and decreased PP1 expression/activity remains to be elucidated, several lines of evidence provide interesting clues. Chronic aluminum exposure impairs long-term potentiation and depression in the rat dentate gyrus *in vivo*, suggesting that aluminum affects both presynaptic and postsynaptic mechanisms of transmission (Chen *et al.* 2002). Interestingly, PP1 is not only highly enriched in dendritic spines (Ouimet *et al.* 1995) and necessary for maintaining long-term depression (Morishita *et al.* 2001), it was also linked to age-related memory and learning deficits (Genoux *et al.* 2002). Thus, although more experimentation is required, it is tempting to hypothesize that the toxic effect of aluminum may be mediated via its effect on PP1.

The mechanisms by which aluminum and other metal neurotoxicants decrease PP1 levels and/or activity have not been addressed, but warrant further experimentation.

Aluminum has not only been implicated in several neurological disorders, but is also known to induce the hyperphosphorylation of neurofilaments and tau proteins (Shin *et al.* 1994; Singer *et al.* 1997), and to promote the aggregation of hyperphosphorylated tau (Shin 2001) and Abeta (A$\beta$) (Ricchelli *et al.* 2005). Aluminum has been a target for research concerning its role as an environmental risk factor for AD, specially since high levels of aluminum were found in AD patient brains (Kruck and McLachlan 1988). A$\beta$ is a 39–43 amino-acid long peptide and the major component of senile plaques in AD brains. It has been suggested that aluminum induces not only the aggregation of A$\beta$, but also its deposition and toxicity (Pratico *et al.* 2002). Interestingly, we have recently reported that A$\beta$ directly inhibits PP1 activity (Vintém *et al.* 2009). Indeed, A$\beta$ peptides specifically inhibit different PP1 isoforms at low micromolar concentrations. The calculated $IC_{50}$ values for A$\beta$1–42 inhibition of PP1 were determined as 2.3 and 1.9 $\mu mol\,l^{-1}$ against recombinant purified PP1$\alpha$ and PP1$\gamma_1$, respectively. As the aggregation state of A$\beta$ is also an important factor for its neurotoxic effects, we also addressed how A$\beta$ aggregation affects PP1 activity. Fibril formation of A$\beta$1–40 and A$\beta$1–42 significantly increased their inhibitory potency against PP1 (up to ninefold higher compared to their oligomeric counterparts), thus making the fibrillar forms even more potent PP1 inhibitors (Vintém *et al.* 2009). Given the reported stimulation of A$\beta$ aggregation by aluminum, it is expected that aluminum might doubly potentiate PP1 inhibition, thus explaining its neurotoxicant properties. Indeed, we have recently shown that aluminum also potentiates the PP1 inhibitory potency of A$\beta$ (A. P. Vintém and E. F. da Cruz e Silva, unpublished results). Although further experimentation is needed to validate our results in clinically relevant samples, decreased PP1$\alpha$ and PP1$\gamma$1 expression was reported in samples from AD patients (Mufson *et al.* 2002). The putative contribution of aluminum to the development of AD may be related to physiological inhibition of PP1 activity, given the ubiquitous expression and wide variety of physiological roles of PP1. The potential usefulness of PP1 for diagnostic purposes needs to be investigated, although it may also prove interesting as a therapeutic target.

## 13.10.7 Conclusions

It has become increasingly clear that protein phosphorylation systems represent potential targets of broad classes of known and suspect neurotoxicants, as indicated by abnormal phosphorylation of key proteins. Inherent to this hypothesis is the widely accepted view that the specificity of signal transduction cascades is integrated through the overlapping and coordinated mechanisms controlling kinases and phosphatases. Surprisingly, to date relatively few studies have addressed the interaction of metals with protein kinases and even fewer with the protein phosphatases. The description of protein phosphatase expression in the nervous system has seen significant progress in recent years and current work is directed to the identification of their regulatory proteins and physiological substrates (Browne *et al.* 2007; Wu *et al* 2007). Many of the pathological effects of metal neurotoxicity in general, including aluminum toxicity, may be explained by metal inhibition of specific protein phosphatases. PP1, a highly abundant phosphatase in dopaminergic neurons of the basal ganglia (da Cruz e Silva *et al.* 1995; Ouimet *et al.* 1995), is inhibited by aluminum and may, therefore, mediate some of the neurotoxic effects of aluminum. By promoting the aggregation of A$\beta$, and consequently increasing its PP1 inhibitory potency, aluminum potentially further enhances the PP1 inhibitory effects.

PP1 (the 'forgetfulness protein') is also highly expressed in the hippocampus (da Cruz e Silva *et al.* 1995; Ouimet *et al.* 1995) and is necessary for maintaining long-term depression (Morishita *et al.* 2001). Our recent work indicates that aluminum-induced neurotoxicity may correlate with the downregulation of the expression and activity of both PP1$\alpha$ and PP1$\gamma$. The specificity of these effects was suggested by their reversal following aluminum withdrawal, at least in cultured cells (Amador *et al.* 2004). Since PP1 is a key player in neuronal signal transduction pathways, such aluminum-induced alterations may lead to an imbalance in protein phosphorylation systems and consequent neurodegeneration. Various interconnected hypotheses have been advanced that underscore different observations as a rationale for the initiation of aluminum neurotoxicity. Although the relation between aluminum and the pathogenesis of neurodegenerative diseases remains controversial, the available evidence supports the 'aluminum hypothesis' (Gupta *et al.* 2005; Kawahara 2005).

Many and varied primary neuronal signaling cascades, in the hippocampus, striatum, and other brain regions, exert their cellular effects by regulation of PP1 activity. Thus, aluminum neurotoxicity may be related to the downregulation of PP1 expression and activity, both directly and via enhanced A$\beta$ aggregation and consequent PP1 inhibition, leading ultimately to abnormal hyperphosphorylation of key neuronal proteins. The potential role of PP1 as a mediator of aluminum neurotoxicity and neurodegeneration is a novel hypothesis that also deserves to be investigated for other metal neurotoxicants. Indeed, besides manganese, which is a known inhibitor of PP1 activity, our recent unpublished work indicates that purified recombinant PP1 is also inhibited by cobalt, nickel, zinc, and lead. Further work is required to determine the physiological relevance of these observations. In conclusion, the study of aluminum neurotoxicity remains topical and is of foremost importance concerning the implications for human exposure.

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