Abstract It is well established that lead (Pb) exposure in humans leads to learning and memory impairment. However, the biological and molecular mechanisms are still not clearly understood. When over activated, serine/threonine protein phosphatases are known to function as a constraint on learning and memory. Activation of these phosphatases can also result in cytoskeletal changes that will adversely affect learning and memory. We investigated the effects of Pb exposure on these phosphatases in primary cultures of human neurons. Neurons were exposed to physiologically relevant concentrations of Pb (5, 10, 20, and 40 µg/dL) and total phosphatase and PP2A activities were determined in neuronal lysate using para-nitrophenyl phosphate (pNPP), and a PP2A-specific phosphopeptide as substrates. Expression of various serine/threonine phosphatases, tau and its phosphorylation state were determined by Western blot (WB) and immunocytochemistry (ICC). We found that the total phosphatase activity in the neuronal lysate was increased by 30–50% by all the concentrations of Pb tested. PP2A activity was increased by 5 µg/dL Pb only. PP1 expression was increased (ranging from 25–50%) by 10, 20 and 40 µg/dL of Pb. PP2B expression was increased substantially (up to 2.5-fold) by 10 µg/dL Pb, whereas, higher concentrations did not show any effect. On the other hand, Pb (at all concentrations used) decreased expression of PP2A and PP5. Pb exposure induced substantial hyperphosphorylation of tau at serine 199/202 by 5 and 10 µg/dL Pb, and Threonine 231 at higher doses. Expression of total tau was mostly unaffected by lead. Immunocytochemistry data confirmed the WB results of expression of PP1, PP2A, tau protein and the phosphorylation of tau. These results support our hypothesis that Pb exposure up regulates some of the serine/threonine phosphatases (PP1 and PP2B) that are known to impair memory formation, and suggest a novel mechanism of Pb neurotoxicity.

Keywords Lead · Tau phosphorylation · Human neuron

Introduction

Lead (Pb) is a toxic heavy metal that can affect several organs including the nervous, hematopoietic, renal, reproductive, endocrine and skeletal systems [1, 2]. The central nervous system (CNS) is particularly sensitive to Pb toxicity and is affected even by low doses [3, 4]. Lead exposure is associated with growth retardation in children [5, 6]. Exposure to low levels of Pb (as low as 7 µg/dL) is associated with impairment of cognitive and behavioural development in children [7, 8] and in young adults [9]. There is strong evidence that low-dose Pb exposure is causally associated with deficits in cognition [10]. An estimated decrease of 4–7 points in the mean IQ score for each 10 µg/dL increase in blood Pb has been reported [10].
Pb preferentially distributes in specific areas of the brain with the highest levels observed in the hippocampus and cerebral cortex—areas that are associated with learning and memory [11]. The exact mechanism(s) by which lead get into the body, and particularly into the brain, is still unknown as no Pb specific transporter(s) has been identified. However, it has been postulated that Pb might use the divalent metal ion transporter 1 (DMT1), which is used by several essential elements [12]. DMT1 is localized in astrocytes and neurons in the normal brain [13–15]. The developing brain is a primary target for lead-induced neurotoxicity. However, the mechanism of lead-induced neurotoxicity and deficits in learning and memory is not well understood. Neurogenesis and synaptogenesis are the main components of learning and memory and these are both known to be affected by Pb [16–19].

Protein phosphorylation/dephosphorylation is one of the key events in the regulation of several cellular events including synaptogenesis. Reversible protein phosphorylation is the result of a delicate coordinated balance between protein kinases and protein phosphatases. The major serine/threonine protein phosphatases in the brain are PP1, PP2A and PP2B [20, 21]. These phosphatases, together with protein kinases, contribute to the control of synaptic plasticity and memory [22–27]. In the last decade, researchers have clearly demonstrated the importance of protein kinases in short and long-term modification of signal transmission between synapses. However, the roles of protein phosphatases have been appreciated recently and need to be further investigated.

Over-expression/over-activation of some of serine/threonine protein phosphatases has been associated with suppression of learning and memory. The role of PP1 in learning and memory has been widely studied [28, 29]. Inhibition of PP1 activity, either genetically or pharmacologically, enhanced learning capacity, suggesting that PP1 is a constraint on memory. This enhancement in learning was correlated with increased phosphorylation of several synaptic and nuclear targets of PP1 including GluR1, CaMKII, and CREB proteins. After learning, inhibition of PP1 prolonged memory, suggesting that PP1 also promotes forgetting. Thus PP1 appears to be a suppressor of learning and memory [30–33]. It has been suggested that PP1 determines the efficacy of learning and memory by limiting acquisition of information and favouring memory decline. When PP1 is inhibited, short intervals between training episodes are sufficient for optimal performance [31]. In addition to PP1, PP2A and PP2B are also known to adversely affect learning and memory [23, 32, 34, 35]. Mice over-expressing calcineurin (PP2B) have normal short-term memory but have profound and specific defect in long-term (hippocampal dependent) memory. The specific defects in long-term memory may be due to a defect in transition between short-term and long-term memory [23]. The effect of Pb exposure on the activity or expression of these phosphatases thus far has not been investigated in humans or in animal models.

An intact microtubule structure is essential for normal neuronal functioning. Disruption of microtubules has been shown to be associated with memory loss and neuronal death [36]. The microtubule network is stabilized by several microtubule-associated proteins (MAP); tau is one of them. Tau can be phosphorylated at several serine or threonine residues [37]. Phosphorylation of tau at a stoichiometry of 2–3 mol phosphates per mole of protein is required for promoting assembly and stability of the microtubule, whereas, its hyperphosphorylation (9–10 mol of phosphate per mole of tau) results in the disruption and disassembly of microtubule and subsequently in memory loss and neuronal death [38–41]. As the phosphorylation state of tau is regulated by the balance between protein kinases and protein phosphatases, over-expression and/or over-activation of phosphatases (PP1, PP2A or PP2B) will affect phosphorylation level of tau and thus the stability of microtubule.

In this study, we tested the hypotheses that exposure of human neurons to Pb results in over-expression or over-activation of serine/threonine protein phosphatases. The results presented here support this hypothesis and suggest a novel mechanism of lead-induced impairment of learning and memory, as these phosphatases are known to directly impair learning and memory.

Materials and Methods

Reagents and Chemicals

All neuronal cell culture media and additives were from Invitrogen (Mulgrave, VIC, Australia) unless otherwise stated. All chemical used for Western blot, immunoprecipitation, PPase activity and immunoblot (unless mentioned) were obtained from Sigma-Aldrich Chemical Co. (Sydney, NSW, Australia). The list of primary antibodies and their sources is given in Table 1. Prof Alistair Sim (University of Newcastle, NSW, Australia) kindly provided the PP1 antibody (PP1c). Secondary anti-mouse and anti-rabbit IgG and anti-rabbit Alexa 594 (Red)-conjugated antibodies were purchased from Molecular probes (Eugene, OR, USA). All commercial antibodies were used at the concentrations recommended by the manufacturer.

Cell Cultures

Human foetal brains were obtained from 16 to 19 week old foetuses collected following therapeutic termination with
informed consent (Ethic approval HREC03187). Primary cultures of human neurons were prepared as described previously [42]. Briefly, cells were plated in 12-well tissue culture plates coated with Matrigel (diluted 1/20 in Neurobasal medium) and maintained in Neurobasal medium supplemented with 2% (v/v) B-27 supplement, 2 mM Glutamax, 50 mM HEPES, 200 IU/mL penicillin G, and 200 µg/mL streptomycin sulphate and 5 mM glucose. Cultures were kept at 37°C/176°C 5% CO₂ in a humidified atmosphere. The medium was changed once to twice per week. We used 3 week-old highly pure neuronal cultures for the experiments.

Preparation of Neuronal Lysate for Western Blots

For Western Blots, cells were cultured in Matrigel coated 12-well plates at a density of 1 × 10⁶ cells per well in supplemented Neurobasal medium (as described above). Neurons were cultured with 5, 10, 20 and 40 µg/dL of elemental Pb for 72 h. A 10 µg/mL Pb solution was prepared by dissolving 183.07 mg of Pb(II) acetate trihydrate (Pb(C₂H₃O₂)₂·3H₂O; FW 379.33) in 10 mL of deionised and sterilized water, taking into account the MW of lead as 207.20. Control cultures were kept in the medium alone. Cells were lysed in 500 µL of RIFA buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 1% NP-40, 5 mM EDTA, 0.5% sod. deoxycholate, 0.1% SDS, 50 nM NaF, 1 mM sodium vanadate, and protease inhibitor cocktail (Roche Diagnostic, Castle Hill, NSW, Australia). The cell lysate was centrifuged at 16,000 g for 10 min. Protein concentration in the supernatant was determined by the Bradford method (Sigma) and stored at −20°C until used.

SDS–PAGE and Immunoblot Analyses

20 µg lysate protein was resolved on 10% SDS–PAGE (NuPAGE 10% from Invitrogen, Carlsbad CA USA) in sample buffer. Protein was transferred onto PVDF membranes; the membranes were blocked with 5% milk in TBS for 1 h and incubated with primary antibody overnight at 4°C. The primary antibodies used are described in Table 1. After incubation, the membranes were rinsed several times, washed with TBS-tween three times (10 min each), and then two times with TBS only. The membranes were then incubated with the secondary antibody for 4 h at RT, washed as before and developed with ECL (Biorad, Gladesville, NSW, Australia) according to the manufacturer instructions. Blots were scanned and quantified using the software ImageJ from NIH (Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, MD, USA, http://rsb.info.nih.gov/ij/, 1997–2007).

Phosphatase Activity Assay

For phosphatase activity, cells were cultured as described above and lysed in the phosphatase activity buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 1% NP-40, 1 mM PMSF, 1 mM Benzamidine, 10 mM beta-mercaptoethanol (βME), and protease inhibitor cocktail. Total phosphatase activity was determined using pNPP (10 mM) as a substrate in 50 mM Tris–HCl, pH 7.4, 0.1 mg/mL BSA, 3 mM CaCl₂, 1 µM calmodulin and 3 mM MnCl₂. The reaction mixture was incubated at 30°C for 30 min and the absorbance measured at 405 nm. PP2A activity was indirectly measured by using 10 nM okadaic acid (OA) in the phosphatase assay buffer and then calculating the OA-inhibited activity.

Direct Estimation of PP2A Activity

Cells were cultured with various concentrations of Pb for 24 h. Cells were harvested and the lysate prepared in 20 mM imidazole–HCl, pH 7.0, 2 mM EDTA, 2 mM EGTA, and the protease inhibitor cocktail. PP2A activity was measured with the PP2A immunoprecipitation phosphatase assay kit (Cat. # 17-313; Millipore, Temecula, CA, USA) according the manufacturer protocol. Briefly, 200 µg neuronal lysate protein was immunoprecipitated with anti-PP2A (clone 1D6) mAb.
1D6) and the activity of the immunoprecipitated PP2A was estimated by colorimetrically measuring the released phosphate from threonine phosphopeptide (K-R-pT-I-R-R) with Malachite Green phosphate detection solution.

Immunocytochemistry

The method was previously described [38]. Human foetal neurons were grown at the density 5 \times 10^6 in Permanox chamber-slides (NUNC) for 2 weeks. Then, cells were fixed with acetone/methanol (vol/vol) for 20 min at −20°C. Cells were then rinsed three times with PBS and a gentle membranous permeabilization was performed by incubation with 0.025% Triton x100 in PBS for 10 min at room temperature. After washing, cells were incubated with 5% normal goat serum (NGS) in PBS for 45 min at room temperature, rinsed twice with PBS and incubated for 1 h at 37°C with selected primary antibodies diluted in 5% NGS. Antibodies used are Tau (for total tau), AT8 and AT-180, anti-PP1, anti-PP2A (clone 1D6). Cells were then washed with 5% NGS solution and incubated for 1 h at 37°C with the appropriate labelled secondary antibodies (Goat anti-mouse IgG or goat anti-rabbit coupled with Alexa 594). Nuclear staining was performed using DAPI at 1 μg/mL for 5 min at room temperature. After several washings with PBS at 37°C, the cover slips were quickly mounted on glass slides with Fluormount-G, and were examined with an Olympus BX60 fluorescence microscope associated with a digital Sensi-Cam. The following three controls were performed for each labelling experiment: (1) isotypic antibody controls for mAbs and serum control for pAbs, (2) incubation with only the secondary labelled antibodies, (3) estimation of auto-fluorescence of unlabelled cells. Fluorescence intensity was quantified using ImageJ 1.38 for Mac OSX (NIH, USA).

Statistical Analyses

Each experiment was repeated at least three times, unless otherwise stated. The number of replicates for each experiment is shown in the figure legends. Student t test of two independent samples with unequal variances was used to measure the significance level of the difference between control and various levels of Pb treatments. Significance level was set at P < 0.05.

Results

Lead Exposure Increases Phosphatase Activity in Human Neurons

To investigate the effect of Pb exposure on phosphatase activity in cultured primary human neurons, we cultured neurons with increasing concentrations of Pb for 72 h and measured phosphatase activity using pNPP as substrate. The results are shown in Fig. 1. All concentrations of Pb tested induced a significant increase (P < 0.05) in total phosphatase activity. The increase in phosphatase activity ranged from 30–46% compared to control levels but was not dose dependent (Fig. 1a). The PP2A activity, which was indirectly calculated as okadaic acid (10 nM)—inhibited activity, was substantially and significantly (P < 0.01) increased (50%) with 5 μg/dL lead. Higher concentrations of Pb (10, 20 and 40 μg/dL) slightly decreased PP2A activity, but the decrease was not statistically significant (Fig. 1b). Direct measurement of PP2A activity with a PP2A activity assay kit, which involves the release of phosphate from threonine phosphopeptide (K-R-pT-I-R-R) by the immunoprecipitated PP2A, revealed similar results. In this case 5 μg/dL Pb significantly (P < 0.05) increased PP2A activity, whereas, higher concentrations of Pb did not significantly affect PP2A activity.

Lead exposure Dysregulates the Expression of Serine/Threonine Protein Phosphatases

We investigated the levels of protein expression of several serine/threonine protein phosphatases in the lysate of Pb-treated neurons. The results of these experiments are shown in Fig. 2. Pb at 10, 20 40 μg/dL significantly increased PP1 expression (P < 0.05); the highest increase (51%) was observed in neurons treated with 10 μg/dL Pb (Fig. 2a). Similarly, a 1.8 and 2.5-fold increase in PP2B expression was seen, respectively, in neurons treated with 5 and 10 μg/dL Pb, however, only the effect of 10 μg/dL Pb was statistically significant (P < 0.02). Higher concentrations did not show any effect on PP2B expression (Fig. 2b). Expression of PP2A was significantly (P < 0.01) decreased by all concentrations of Pb tested (Fig. 2c). PP5 expression was also decreased by Pb in a dose-dependent manner.
Pb at 5 µg/dL decreased PP5 expression but the decrease was statistically not significant ($P = 0.09$). Higher concentrations of Pb significantly decreased PP5 expression ($P < 0.01$). PP5 was more sensitive to Pb than PP2A. Pb at 40 µg/dL decreased PP5 expression to less than 20% of control. The increase in PP1 expression and the decrease in PP2A expression with Pb treatments were confirmed with immunocytochemistry (Fig. 4).

**Discussion**

We found that Pb in pathophysiological relevant concentrations increased total phosphatase activity and PP2A activity in primary cultures of human neurons. Pb also resulted in increased expression of PP1 and PP2B but it decreased the expression of PP2A and PP5. Pb substantially increased phosphorylation of serine 199/202 of tau (AT-8 site) particularly at lower doses (5 and 10 µg/dL), whereas, phosphorylation of threonine 231 was more pronounced at higher dose (40 µg/dL). These results suggest two possible mechanisms for Pb-induced impairment of learning and memory. First, Pb treatment (exposure) induces over-expression (PP1 and PP2B) and over-activation (PP2A) of serine/threonine protein phosphatases. Over-activation of these phosphatases is associated with learning and memory impairment [28–35]. Second, Pb exposure results in hyperphosphorylation of tau which may subsequently result in cytoskeletal disruption in neurons. Such structural changes are also reported to be responsible for memory loss in many tauopathies [38–41].

Our study is unique in three aspects. Firstly, we measured the activity and expression of PP1, PP2A and PP2B in primary cultures of human neurons. To our knowledge,
This is the first study investigating the mechanism of Pb-induced changes at the molecular level in human neurons. Previous studies have been performed using animal models; however, these results may not be fully relevant for humans. Secondly, the effects of Pb exposure on brain phosphatases have not been studied before in humans or in animal models. Finally, we used pathophysiological levels of Pb that are in accordance with the blood levels of humans that have been exposed to environmental Pb contamination.

We found a 30–46% increase in total phosphatase activity in neurons treated with Pb for 72 h. Since PP2A accounts for the majority (~70%) of the brain phosphatase activity, we used two approaches to measure the activity of this phosphatase. (1) Okadaic acid at 10 nM concentration specifically inhibits PP2A activity without affecting PP1 or PP2B activity [43]. We used this approach to indirectly estimate PP2A activity. Using this method, we found that PP2A activity was particularly sensitive to Pb effect and we observed a significant increase (50%) but only with a small dose of Pb (5 µg/dL). Higher doses were not effective and resulted only in a limited (non-significant) decrease in PP2A activity. (2) A commercially available kit (Millipore, Temecula, CA, USA), which specifically measure PP2A activity was used. This technique involves the immunoprecipitation of PP2A with the anti-PP2A antibody (clone 1D6) and measuring the released phosphate by PP2A colorimetrically from a phosphopeptide. The results obtained from this approach were in perfect agreement with the okadaic acid-inhibited activity (Fig. 1b, c). From these results it can be confidently inferred that PP2A is more sensitive to lower doses of Pb and that such increase in PP2A activity by low doses of Pb may responsible for reported CNS toxicity [3, 4] and impairment of learning and memory [10] by low doses of Pb.

Several serine/threonine protein phosphatases are expressed in the brain. Of these, the major phosphatases in the brain are PP1, PP2A and PP2B [20, 21], with PP2A being the major one. PP2A (together with PP1) accounts
for over 90% of the total mammalian brain protein phosphatase activity [44, 45]. PP2A alone also accounts for 71% of the brain tau phosphatase activity [46]. Our results suggest that different phosphatases are affected differently by Pb. We observed an increase in PP1 and PP2B expression but a decrease in the expression of PP2A and PP5. The increase in PP1 and PP2B expression may be a mechanism to compensate for the decrease in PP2A expression. Our results also suggest that the increase in total phosphatase activity, particularly at Pb doses higher than 5 μg/dL may be mainly due to an increase in PP1 and PP2B activity. However, this could not be verified as we did not individually determine the activities of PP1 and PP2B.

Treatment with low-level of Pb (5 μg/dL) resulted in increased activity of PP2A but a decrease of its expression. The reason for this apparent discrepancy between activity and expression of PP2A by Pb is not clear but could likely be explained by the complex structure and regulatory system of PP2A. PP2A is a trimeric protein consisting of a catalytic subunit C, a scaffolding subunit A and a regulatory subunit B. There are two isoforms of C, two isoforms of A and at least 18 isoforms of the regulatory subunit B. The expression levels of the A and C subunits are uniform throughout the brain but the expression levels of various regulatory subunits are highly variable depending upon cell types and tissues [47, 48]. Different combinatorial associations of the different A, B and the C subunits can generate 75 different trimeric PP2A holoenzymes. The activity of the holoenzyme towards different substrates is greatly modulated by the regulatory subunit B. These may modulate (1) the tissue localization and targeting the enzyme to particular cellular compartments, (2) the substrate specificity, and (3) catalytic activity toward the same substrate in response to different agents [47]. The C subunit as such is more active towards phosphoprotein substrates than is the core enzyme (Subunits A and C) in vitro, whereas it is the opposite for activity towards phosphopeptides [49, 50]. Similarly, the B subunits also modulate the catalytic activity of the core enzyme, suppressing activity towards some substrates, but greatly enhancing the dephosphorylation of others (reviewed by Janssens and Goris [47]).

Whether Pb affects the association of the catalytic subunit of PP2A with other subunits or results in the selective expression of different regulatory subunits needs to be further investigated.

In addition to the direct impairment of learning and memory due to increase in the activity of phosphatases, Pb may also indirectly impair learning and memory by causing cytoskeletal changes in neurons by modulating the
phosphorylation state of the tau protein. We observed a substantial increase in tau phosphorylation at two sites (serine 199/202 and threonine 231) in human neurons treated with various concentrations of Pb. An increase in the expression of phosphorylated tau in the hippocampus of mouse pups exposed to Pb in their early life has recently been reported [51]. Tau is a phosphoprotein that can be phosphorylated at multiple serine and threonine residues. Hyperphosphorylated tau is assembled into paired helical filaments (PHF) of neurofibrillary tangles [52, 53]. At least 30 serine/threonine phosphorylation sites have been identified in abnormally hyperphosphorylated tau [37]. Phosphorylation of tau at a stoichiometry of 2–3 mol phosphates per mole of protein is required for promoting assembly and stability of microtubule, whereas, its hyperphosphorylation (9–10 mol of phosphate per mole of tau) results in the disruption and disassembly of microtubule and subsequently in memory loss and neuronal death [36, 37, 41]. The disruption of microtubules in neurons by the hyperphosphorylated tau is one of the fundamental aspects of dementia in Alzheimer’s disease and other tauopathies [52–57]. Whether tau phosphorylation is involved in Pb-induced deficits in learning in young children is still unknown, and needs to be further investigated. However, several studies have shown that phosphorylated tau, irrespective of NFT accumulation, is responsible for memory deficits in many models of tauopathies (other than Alzheimer’s disease) such as Drosophila [58], mouse [59] and rat [60]. Based on these reports it would not be surprising if such mechanism also affect young children exposed to lead. The increase in tau phosphorylation at low dose of lead (5 µg/dL), particularly at serine 199/202 may be a mechanism for the reported impairment of learning and memory in children. This finding is in accordance with the published literature showing that Pb even at low doses (as low as <7 µg/dL) causes subtle alteration in synaptogenesis and impairs learning and memory, whereas, at high doses, it can cause encephalopathy [16, 61, 62].

In summary, we report here that Pb exposure, in pathophysiological concentrations, increases the activity and expression of different serine/threonine protein phosphatases. Pb exposure also induces hyperphosphorylation of
tau at several sites. These data suggest that Pb may impair learning and memory by two mechanisms: (1) by up-regulating the activity (PP2A and possibly PP1 and PP2B) and expression (PP1 and PP2B) of protein phosphatases, which are known to impair learning and memory formation and (2) by inducing hyperphosphorylation of tau protein and the subsequent cytoskeletal changes in neurons.

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