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Increased β -amyloid Levels in the Choroid Plexus Following Lead Exposure and the Involvement of Low Density Lipoprotein Receptor Protein-1

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Abstract

The choroid plexus, a barrier between the blood and cerebrospinal fluid (CSF), is known to accumulate lead (Pb) and also possibly function to maintain brain's homeostasis of A β , an important peptide in the etiology of Alzheimer's disease. This study was designed to investigate if Pb exposure altered A β levels in the blood-CSF barrier in the choroid plexus. Rats received ip injection of 27 mg Pb/kg. Twenty-four hr later, an FAM-labeled A β (200 pmol) was infused into the lateral ventricle and the plexus tissues removed to quantify A β accumulation. Results revealed a significant increase in intracellular A β accumulation in the Pb-exposed animals compared to controls ($p < 0.001$). When choroidal epithelial Z310 cells were treated with 10 μ M Pb for 24h and 48h, A β (2 μ M in culture medium) accumulation was significantly increased by 1.5 fold ($p < 0.05$) and 1.8 fold ($p < 0.05$), respectively. To explore the mechanism, the effect of Pb on low-density lipoprotein receptor protein-1 (LRP1), an intracellular A β transport protein, was examined. Following acute Pb exposure with the aforementioned dose regimen, levels of LRP1 mRNA and proteins in the choroid plexus were decreased by 35 % ($p < 0.05$) and 31.8% ($p < 0.05$), respectively, in comparison to those of controls. In Z310 cells exposed to 10 μ M Pb for 24 h and 48 h, a 33.1% and 33.4% decrease in the protein expression of LRP1 was observed ($p < 0.05$), respectively. Knocking down LRP1 resulted in even more substantial increases of cellular accumulation of A β , from 31% in cells without knockdown to 72% in cells with LRP1 knockdown ($p < 0.05$). Taken together, these results suggest that the acute exposure to Pb results in an increased accumulation of intracellular A β in the choroid plexus; the effect appears to be mediated, at least in part, via suppression of LRP1 production following Pb exposure.

Keywords

blood-CSF barrier; Pb; A β ; LRP1; choroid plexus; blood-brain barrier; Alzheimer's disease

Introduction

Lead (Pb)-induced neurotoxicity remains a major public health concern not only in developing countries but also in developed countries (Davis et al., 1993; Dingwall-Fordyce.,1963; Goyer et al., 1993;). As an indispensable metal used in the modern industry, the demand for Pb has

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been steadily increasing in the last decade. Despite its occupational hazard, environmental exposure to Pb in the general population continues to be a major public health issue, as the metal is widely present in air, drinking water, household products, plastic materials, paints, and other products (Staudinger et al., 1998).

While a definite relationship between Pb exposure and the pathogenesis of Alzheimer's disease (AD) has yet to be established, some reports have suggested that Pb may be a risk factor in AD. For example, Graves et al. (1991), by re-analyzing four case-control studies, revealed a positive correlation between Pb exposure and AD. Haraguchi et al. (2001) showed the presence of high levels of Pb in diffuse neurofibrillary tangles, a form of pre-senile dementia, in 10 AD cases compared with 9 controls. More recent studies on Pb-exposed workers demonstrate an exacerbated neurodegeneration and atrophy in brain regions that are similar to those seen in AD patients (Jiang et al., 2008; Stewart et al., 2006). Pb has long been shown to affect memory in children (Counter et al., 2005) and development in animals. For example, studies conducted on mice and non-human primates suggest that Pb exposure during early development alters the expression and regulation of amyloid precursor protein (APP) with an increased aggregation of A β later in life (Basha et al., 2005a, b; Wu et al., 2008) and impairs certain forms of memory (Kuhlmann et al., 1997). Thus, the linkage between Pb exposure and AD etiology, particularly in disruption of A β metabolism in the brain, deserves further exploration (Prince 1998; White et al., 2007).

Accumulation of A β in brain extracellular space is often considered one of the major hallmarks of AD pathogenesis (Ogomori et al., 1989). Increased levels of A β in the brains of AD patients may occur by one or more processes, including overproduction of A β in the brain, inadequate metabolic clearance within the brain, or a disrupted transport of A β into and out of brain by the brain barrier system. There are two brain barriers that separate the brain parenchyma from the blood circulation. The blood-brain barrier (BBB), which is mainly composed of tightly connected cerebral capillary endothelia, separates the blood from the brain interstitial fluid. The barrier between the blood and the cerebrospinal fluid (CSF), known as the blood-CSF barrier (BCB), is located in the choroid plexus. Since there is no barrier between brain interstitial fluid and the CSF, A β in the brain extracellular space can readily enter into the CSF (Brody et al., 2008). In fact, the CSF concentration ratio of two major A β peptides (i.e., A β ₁₋₄₀ and A β ₁₋₄₂) has been suggested as a biomarker for AD diagnosis (Kanai et al., 1998). Noticeably, A β ₁₋₄₀, a soluble form of A β peptides, is the major component of diffused plaques in AD patients (Mehta et al., 2000; Seubert et al., 1992; Vigo-Pelfrey et al., 1993). The regulation of A β at the BBB has been previously reported (Deane et al., 2004; Donahue et al., 2006). Although A β has been detected in the choroid plexus of AD patients (Kalaria et al., 1997; Miklossy et al., 1999), the role of the BCB in A β transport and metabolism remains unclear.

Available data in literature suggest that low-density lipoprotein receptor protein 1 (LRP1) may play a role in A β export from the brain to the blood by crossing the BBB (Goto and Tanzi, 2002; Knauer et al., 1996; Kounnas et al., 1995; Moir and Tanzi, 2005). LRP1 is a 600 kDa trans-membrane glycoprotein and is involved in receptor-mediated endocytosis and cell signaling (Hyman et al., 2000). It is subsequently cleaved by furin into an external 515 kDa α subunit and an 85 kDa β subunit which contains a transmembrane domain and a cytoplasmic tail with two NPXY motifs. These two motifs, which contain numerous tyrosine residues (Herz et al., 2001), can interact with signaling proteins and are known to participate in clearing A β via the BBB (Harris-White et al., 2005; Kounnas et al., 1995). At the BBB, A β molecules in the brain extracellular fluid can be taken up by known transport mechanisms to brain endothelial cells (Deane et al., 2004). Within the cells, the binding of A β to LRP1 allows the LRP1 to carry A β molecules to the basolateral inner membrane facing the blood with the subsequent expelling of A β into the blood stream (Deane et al., 2004; Donahue et al., 2006).

Interestingly, A β has also been shown to promote proteasome induced degradation of LRP in the endothelium of the BBB, subsequently resulting in decreased LRP expression, consistent with reduced LRP levels in both patients suffering from AD and in A β accumulating transgenic mice (Deane et al., 2004; Deane et al., 2008). In addition, LRP1 has been found capable of binding to A β ligands like apolipoprotein E, APP and α 2-macroglobulin (Kang et al., 2000; Knauer et al., 1996; Kounnas et al., 1995; Uden et al., 1999; 2000) thereby promoting A β clearance. Thus, LRP1, by either directly transporting A β or binding to these important proteins in regulating A β homeostasis, may mediate the clearance A β from the brain (Herz et al., 2001). Although, LRP1 has been recently identified in the choroid plexus (Johanson et al., 2006), its role in the BCB still largely remains unknown.

Human autopsy data and animal studies from this and other laboratories have established a clear relationship between Pb exposure and the ensuing accumulation of Pb in the choroid plexus (Friedheim et al., 1983; Manton et al., 1984; O'Tuama et al., 1976; Zheng et al., 1991, 1996). Accumulation of Pb in the choroid plexus has been shown to increase the leakage of the BCB (Shi and Zheng et al., 2007) and alter the production of transthyretin (TTR) and transport of thyroxine by the BCB (Zheng et al., 1996, 2001, 2003). Previous research in our laboratory has also shown that A β ₁₋₄₀ is actively transported by the choroid plexus, predominantly from the CSF towards the blood (Crossgrove et al., 2005). Since Pb exposure results in its accumulation in the choroid plexus and since A β is extensively transported by this tissue, it became interesting to investigate whether Pb accumulation in this barrier tissue altered the clearance of A β from the CSF, which may contribute to the etiology of AD.

This study was designed to test the hypothesis that acute exposure to Pb interfered with A β ₁₋₄₀ regulation in the choroid plexus and this interference may result from Pb altering A β uptake and/or clearance via LRP1. An acute Pb exposure model with an intraperitoneal (ip) injection of 50 mg Pb-acetate/kg (i.e., 27 mg Pb/kg) was used because our previous studies using the same dose regimen have shown a substantial accumulation of Pb in the choroid plexus (Zheng et al., 1991). The purpose of this dose regimen was not to mimic real life exposure, but instead, to produce a condition in which the amount of Pb in the choroid plexus could build up significantly during a fairly short period of time.

Materials and Methods

Materials

Chemicals and assay kits were purchased from the following sources: FAM-labeled A β (catalog # 23514-01) from Anaspec (San Jose, CA), SOD assay kit (catalog # K335-100) from Biovision (Mountain View, CA), ELISA kit (catalog # KHB-3481) and ultra purified A β (1-40) (catalog # 03-138) from Biosource (Carlsbad, CA), rabbit anti LRP1 antibody (catalog # ARP32793) from Aviva (San Diego, CA), Alexa-labeled secondary antibody from Molecular Probes (Eugene, OR), enhanced chemiluminescence reagent (ECL) and ECL films from Amersham Biosciences (Piscataway, NJ), Dulbecco's modified essential medium (DMEM), fetal bovine serum (FBS), penicillin and streptomycin, gentamycin from Gibco (Grand Island, NY), the PCR buffer, dNTP, Oligo dT and MuLV reverse transcriptase from Applied Biosystems (Foster City, CA), LDH assay kit (catalog # TOX-7), β -actin, dithiothreitol (DTT), 2-mercaptoethanol, phenylmethylsulfonyl fluoride (PMSF), polyacrylamide, tetramethylethylenediamine (TEMED), and the siRNA for LRP1 and all other chemicals were from Sigma Chemicals (St. Louis, MO). TRIzol was purchased from Invitrogen (Carlsbad, CA), oligodT, MuLV from Applied Biosystems (Foster City, CA), the ABsolute QPCR SYBR green Mix kit from ABgene (Rochester, New York), the primers from Integrated DNA Technology Inc. (Coralville, IA), and transfection agent, lipofectamine from Ambion (Austin, TX). All reagents were of analytical grade, HPLC grade or the best available pharmaceutical grade.

Animals and treatment

Male Sprague-Dawley rats at the time they were used were 8–9 weeks old (250–300g). The animals were housed in a temperature-controlled, 12:12 light/dark room, and were allowed free access to tap water and food. Rats received an ip injection of 50 mg/kg Pb acetate (i.e., 27 mg Pb/kg) or an equivalent molar concentration of Na-acetate (i.e., 15 mg acetate/kg) as controls. Twenty-four hours post injection the rats were anesthetized with ketamine/xylazine (75:10 mg/mL, 1 mL/kg body weight), immobilized in a stereotaxic device and subjected to the following experimentation. Animal protocols pertinent to this study were approved by the Purdue University Animal Care and Use Committee.

Intraventricular perfusion of A β

A midline cutaneous incision was made from the head to the neck on the dorsal surface to expose the skull. A hole was drilled in the skull at co-ordinates determined using the Paxinos and Watson Atlas at 0.8 mm posterior to bregma and 1.4 mm lateral) followed by an insertion of a sterilized cannula at 3.5 mm ventral from the skull surface. An internal cannula connected to PE 50 tubing was inserted into the guide cannula for lateral ventricle perfusion. The other end of the PE tubing was attached to a 10 μ L Hamilton syringe, which was filled with 200 pmoles of FAM-labeled A β diluted in artificial CSF (Yamada et al., 1998).

The A β solution was infused into the lateral ventricle at a rate of 12 μ L/min for 0.5 min. The cannula was allowed to remain inside the ventricle for an additional 0.5 min before it was removed in order to avoid the back flow into the tubing. Twenty min post infusion, rats were euthanized with ketamine-xylazine and the brain was dissected to remove the choroid plexus. The time of A β incubation (15–20 minutes) was based on previous studies, which establishes a clearance of A β by the BBB within 30 minutes (Bell et al., 2007). The tissues were then transferred to a 35-mm dish and washed three times with an artificial CSF (aCSF). Aliquots (1–2 drops) of aCSF were then added on the tissue to prevent it from drying out; the tissue was observed immediately using an inverted laser scanning microscope (Olympus, FV1000) for live uptake.

Confocal immunofluorescence microscopy

To acquire images, the 35mm dish containing the choroid plexus specimen in artificial CSF was mounted on the stage of an Olympus, FV1000 inverted confocal laser-scanning microscope and viewed through a 40x water-immersion objective (numeric aperture=1.2), with a 488-nm laser line for excitation (Ar-ion laser). Low laser intensity was used to avoid photo bleaching. The choroid plexus was examined under reduced transmitted-light illumination and an area containing undamaged epithelium with underlying vasculature was selected. Each sample was imaged at a rate of one frame per second and care was taken to expose all the tissues for the same period of time at a reduced illumination setting to avoid photo bleaching. Confocal images (512 $\times$ 512 $\times$ 8 bits, 4 frames averaged) were acquired and saved to a disk. For each tissue sample, 4 areas of cells were selected for image collection.

The fluorescence intensity was further quantified using software ImageJ and reported in arbitrary units (a.u). Data reported, unless otherwise stated, are the results of single experiments representative of three to four replicate experiments.

Culture of choroidal epithelial Z310 cells

The characteristics of immortalized rat choroidal epithelial Z310 cells have been described in a previous publication (Zheng and Zhao, 2002). Briefly, cells were maintained in DMEM (high glucose) medium supplemented with 10% FBS, 100 U/mL penicillin, 100 μ g/mL streptomycin,

and 40 µg/mL of gentamycin in a humidified incubator with 95% air–5% CO₂ at 37°C and were passaged twice a week.

Determination of Pb-induced cellular toxicity in Z310 cells

To choose the Pb concentration at which it altered Aβ transport by the choroid plexus but did not induce nonspecific cytotoxicity, three general cytotoxicity assays were used, including the methylthiazolyldiphenyl-tetrazolium bromide cell viability assay (MTT), cell membrane permeability assessment (lactate dehydrogenase or LDH assay), and cellular oxidative stress estimation (superoxide dismutase or SOD assay). MTT cell viability assay was performed by growing Z310 cells at a density of 40,000 cells/well for 2–3 days until they reached 80–85% confluence. The medium was then removed and replaced with fresh medium containing different concentrations of Pb as Pb-acetate (0, 5, 10, 25, and 50 µM). The cells were incubated for an additional 24 h, followed by adding an aliquot of MTT stock solution (2 mg/mL in PBS) to each well. The absorbance of the converted dye was measured at a wavelength of 570 nm. To determine the LDH activity, Z310 cells were treated in the same way as described in MTT assays. An LDH assay was then conducted using a LDH assay kit as per protocol. The SOD activity was determined according to the instruction of the assay kit. The cells were treated with Pb at 0 or 10 µM for 24 h.

Detection of Aβ_{1–40} in Z310 Cells following Pb exposure by immunofluorescence

The effects of Pb exposure on Aβ uptake in choroidal epithelial cells were qualitatively assessed using confocal microscopy. Z310 cells were plated on 35-mm glass plates (MatTek, Ashland, MA) and grown until they reached about 85% confluence (2–3 days). The cells were treated with 10 µM Pb as Pb acetate for 24 h in DMEM medium. Following exposure, the cells were washed 3 times with phosphate buffered saline (PBS) to remove all remnants of Pb in the dish. Cells were then incubated with 200 µL of 2 µM fluorescent labeled Aβ for one hour followed by three PBS washes. Fresh cell culture medium was then added and the preparations were observed under a confocal microscope.

Quantification of Aβ_{1–40} accumulation in Z310 cells following Pb exposure by ELISA

Intracellular accumulation of Aβ in Z310 cells following Pb exposure was further quantified using a well established enzyme linked immunosorbent assay (ELISA) After Pb exposure at 10 µM for 4, 12, 24 or 48 h, Z310 cells were washed with PBS and incubated with 200 µL of a 2 µM solution of unlabeled ultrapure Aβ_{1–40} in serum free medium for 1 h. Cells were washed 3 times with PBS to remove excess Aβ, collected and sonicated to lyse the cells. The cell lysates were diluted 3:1 with diluent buffer (as per manufacturer's instructions) before adding them to the ELISA plates. Aβ_{1–40} colorimetric kit (Invitrogen KHB3481) was used to determine the concentrations of Aβ_{1–40} in the cell lysates. This assay kit detects monomeric Aβ_{1–40} since this form is more likely to be transported across the barrier rather than the aggregated form. An aliquot of cell lysates was used to quantify the protein concentrations by the Bradford assay to normalize the Aβ_{1–40} to total protein.

Quantification of LRP1 mRNA expression by real-time RT-PCR

The transcription of the gene encoding LRP1 was quantified using real-time RT-PCR as described by Walker et al., (2001). Briefly, total RNA was isolated from Z310 cells or choroid plexus tissue using TRIzol reagent following the manufacturer's directions. An aliquot of RNA (1 µg) was reverse-transcribed with MuLV reverse-transcriptase and oligo dT primers. The forward and reverse primers for target genes were designed using Primer Express 3.0 software. The ABsolute QPCR SYBR green Mix kit (ABgene, Rochester, New York) was used for real-time RT-PCR analyses. The amplification was carried out in the MX 3000P real-time PCR System (Stratagene, La Jolla, CA). Amplification conditions were 15 min at 95 °C, followed

by 40 cycles of 30 s at 95 °C, 1 min at 55 °C and 30 s at 72 °C. A dissociation curve was used to verify that the majority of fluorescence detected could be attributed to the labeling of specific PCR products, and to verify the absence of primer-dimers and sample contamination.

All real time RT-PCR reactions were done in triplicate. Primers sequences for rat LRP1 used for real-time RT-PCR were: forward primer 5'-TTGTGCTGAGCCAAGACATC -3' and a reverse primer 5'GGCGTGAAGACATGTAGGT -3' (Genbank Accession No XM_243524) and rat glyceraldehydes-3-phosphate dehydrogenase (GAPDH), used as an internal control, had a forward primer 5'-CCT GGA GAA ACC TGC CAA GTA T-3' and a reverse primer 5'-AGC CCA GGA TGC CCT TTA GT-3' (Genbank Accession No. NM_017008).

Quantification of LRP1 protein expression by Western blot

The choroid plexus tissues or Z310 cells were homogenized (1:10, wt/vol) on ice in a buffer containing 20 mM Tris (pH 7.5), 5 mM EGTA, 1% TritonX-100, 0.1% SDS, 50μM phenylmethylsulphonylfluoride (PMSF), 15 mM 2-mercaptoethanol and a Protease Inhibitor Cocktail containing 500 μM 4-(2-Aminoethyl) benzenesulfonyl fluoride hydrochloride (AEBSF), 150 nM aprotinin, 1 μM E-64, 0.5 mM EDTA, 1 μM leupeptin (Calbiochem, San Diego, CA). Samples were sonicated using a Model 500 Sonic Dismembrator (Fisher Scientific) at duty cycle 20% and output 4–6 for 30 pulses. Following centrifugation at 10,000 g at 4°C for 10 min, aliquots of supernatants were assayed for protein concentrations by the Bradford method. A volume of protein extract (40 μg of protein) was mixed with an equal volume of 2X sample buffer (0.35 M Tris-Cl, 10% SDS, 30% glycerol, 0.6 M DTT, and 0.012% bromophenol blue), loaded onto a 10% SDS-polyacrylamide gel, electrophoresed, and then transferred to a PVDF membrane. The membrane was blocked with 5% dry milk in TBST (Tris-buffered saline) at room temperature for 1 h and immunoblotted with an antibody directly against LRP1 (1:250). This antibody, purchased from Aviva Systems Biology (accession number Q6PJ72), identifies the swissprot ID of LRP1 at 48 kD. The membrane was stained with a horse-radish peroxidase (HRP)-conjugated goat anti-rabbit IgG antibody (1:5000) at room temperature for 1 h and developed using ECL reagent and films. The exposure time varied from 30 sec to several min depending on signal strength. β-actin (42 kD) (1:2000) was used as an loading control; the corresponding secondary antibody (1:2000) for β-actin was HRP-conjugated goat anti-mouse IgG. Band intensities were quantified using Scion Image software (Frederick, Maryland) and results were reported as a ratio of LRP1 to β-actin in the tissue or cells.

Aβ accumulation following LRP1 knockdown by RNAi

siRNA Transfection was performed as follows: Candidate sequences for LRP1 knockdown were obtained commercially from Sigma-Aldrich. The sequences used were- Forward primer: 5' CCUAUCUUUGAGAUCGAA 3'; reverse Primer: 5' UUCGGAUCUCAAGAUAGG 3. Transfection agent lipofectamine was found to work best in the Z310 cells after a series of screening. Transfection conditions were optimized according to the following variables" initial seeding density, volume of transfection agent, duration of transfection, concentration of siRNA.

Cells were seeded at a density of 1×10^5 cells/well in a 6- well plate in cell culture medium. After 24 hours, the RNA/transfection system was prepared as follows: 1 ul lipofectamine was diluted in 100 uL OPTI-MEM 1 medium and incubated for 10 minutes at room temperature. siRNA was added to a separate 100 uL OPTI- MEM medium to obtain a final concentration of 50 nM per dish. The transfection agent and the siRNA were then mixed and incubated at room temperature for 45 minutes with occasional mixing. Cell culture medium was replaced with 200ul of the above mixture along with 800 uL of OPTI-MEM medium to obtain a total of 1 ml medium/well for 5 hours. An additional 1 ml of regular cell culture medium was added

and the cells were grown for an additional 48 hours. The cells were transfected with either scrambled siRNA as a negative control or the siRNA sequence designed homologous to LRP1. A negative control was used to demonstrate that there was no non-specific toxicity caused by the transfection agent. Knockdown was then analyzed by laser scanning cytometry, real time RT-PCR and western blot analysis. Cells were exposed to Pb at 10 μ M for 24 hours. A β uptake studies were performed as described earlier using ELISA and intracellular A β was normalized to total protein. The intracellular A β levels were normalized by the cellular total protein concentrations.

Statistical analysis

Statistical analyses of the differences between groups were carried out by a one-way ANOVA with post hoc comparisons by the Dunnett's test or using paired t-tests (Kaleidagraph 3.6) and by using SPSS (version 30.0) to determine correlation coefficients. All data are expressed as mean $\pm$ SD. Differences between two means were considered significant when *p* was equal or less than 0.05.

Results

Increase in accumulation of intracellular A β_{1-40} in rat choroid plexus tissue following acute Pb exposure

In the current study, rats received a single ip injection of 50 mg/kg of Pb acetate (27 mg/kg Pb). The purpose of this dose regimen was to produce a significant buildup of Pb in the choroid plexus during a short period of time, as our previous study has shown that the same dose regimen produces a marked accumulation of Pb in the choroid plexus (5.8 μ g/g of tissue) that is 12-fold greater than in the brain cortex (Zheng et al., 1991). Following an intraventricular infusion of FAM-labeled A β_{1-40} , the fluorescent signals were evident in choroid plexus tissues (Fig. 1A); this observation confirmed our previous report that the choroid plexus possesses the capacity to acquire A β from the CSF (Crossgrove et al., 2005). Remarkably, Pb-treated animals showed much more abundant fluorescent labels in plexus tissues than did the controls (Fig. 1B). Moreover, the A β_{1-40} derived signals appeared to be mainly distributed in the choroidal epithelial cytoplasm but not within the nuclei in both control and Pb exposed groups. Quantification of the fluorescence by laser scanning cytometry using software Image J revealed a highly significant difference between the Pb-exposed group (342.3 $\pm$ 49.4) and the controls (119.1 $\pm$ 31.9) (*p*<0.001) (Fig. 1C). Fluorescence was expressed in arbitrary units (a.u.).

Cytotoxicity assays to determine the concentration of Pb in Z310 cells

To ensure that the Pb effect on A β in our subsequent in vitro studies was not due to a direct Pb-induced cytotoxicity, we first set out to screen an appropriate Pb concentration. As shown in Table 1, the MTT cell viability assay with Pb concentrations ranging between 0–50 μ M revealed that exposure with 10 μ M Pb yielded 96.3 % viable cells, which was not significantly different from controls. The LDH assays revealed that Pb concentrations at or below 10 μ M had no significant effect on LDH release. Results from the SOD assay further showed that there was no significant oxidative stress generated by Pb at 10 μ M. Based on these findings, along with previously published data from this group (Shi et al., 2007), a concentration of 10 μ M Pb was chosen for the following A β studies.

Increase in accumulation of intracellular A β_{1-40} in Z310 cells following acute Pb exposure

Prior to testing A β accumulation following Pb exposure in Z310 cells, a MTT cell viability assay was also performed to test the toxicity of A β to Z310 cells. Results revealed 90% viability in cells exposed to 2 μ M A β for 1 h compared with untreated cells indicating that A β at this concentration was not toxic to the cells. Z310 cells were incubated with 2 μ M A β in culture

medium for 1 h and observed for A β derived fluorescent signals within the cells. Data in Fig. 2A demonstrated that normal Z310 cells had the capacity to take up A β . After the cells were exposed to 10 μ M Pb for 24 h or 48 h, followed by incubation with 2 μ M A β in the culture medium for another one hour, a stronger A β signal in the Pb-treated cells was observed when compared to the controls at 24 h (Fig. 2B). The elevation in A β signals persisted at 48 h following Pb treatment (Fig. 2C). Similar to results obtained from in vivo studies, the A β signals were primarily localized in the cytosol but not in nuclei.

The dose-time dependence of Pb on A β accumulation in Z310 cells was further investigated using an established ELISA assay. Following exposure to 0, 1, 2.5, 5, or 10 μ M of Pb for 24 h, the amount of A β in Z310 cells appeared to be increased with increasing Pb concentrations in the range of 1–10 μ M, except for Pb concentration at 2.5 μ M (Fig. 3A). Exposure to 10 μ M Pb produced a statistically significant increase of A β in the Z310 cells ($r = 0.49$, $p < 0.05$). A time-course study showed a continuous increase in intracellular A β after the cells were exposed to 10 μ M Pb for 4, 12, or 24 h (Fig. 3B). Even at 48 h, A β accumulation in Z310 cells was significant, about 1.8 fold higher as compared to control ($r = 0.9$, $p < 0.05$).

LRP1 mRNA expression was decreased in vivo and in vitro following acute Pb exposure

To examine the mechanism by which Pb exposure retained intracellular A β , we quantified the mRNA expression of LRP1 as the consequence of Pb exposure by real time RT-PCR. The amount of mRNA was normalized using the housekeeping gene, GAPDH. Following in vivo Pb exposure (27mg/kg i.p), a significant decrease of LRP1 mRNA expression (–31.8%) in the choroid plexus was observed in comparison to controls ($p < 0.05$, Fig 4A). In vitro exposure of Z310 cells to Pb at 10 μ M for 24h also resulted in a significant reduction (–41.1%) in LRP1 mRNA expression as compared to controls ($p < 0.05$, Fig 4B). This reduction in LRP1 mRNA persisted even at 48 h after Pb exposure ($p < 0.05$).

LRP1 protein expression was decreased following in vivo or in vitro Pb exposure

Western blot analysis by antibodies against LRP1 was used to quantify the effect of acute Pb exposure on LRP1 protein expression. Our results demonstrated that the protein concentrations of LRP1 in the choroid plexus were significantly lower in rats receiving acute Pb exposure (27mg Pb/kg ip for 24 hr) than those in controls (–35%) ($p < 0.05$, Fig 5A, B). In vitro exposure of Z310 cells to 10 μ M Pb for 24h and 48h, revealed a significant decrease of 33.1 % and 33.4% respectively in LRP1 protein expression compared to controls ($p < 0.05$, Fig 5C, D).

Intracellular A β 1–40 accumulation in Z310 cells was increased following LRP1 knockdown by siRNA

To test whether the accumulation of A β following Pb exposure was indeed mediated by LRP1 in the BCB, we assessed A β accumulation in Z310 cells following LRP1 knockdown by siRNA. The system was first optimized using real time RT-PCR, Western blot and laser scanning cytometry to determine an optimum concentration of the LRP1 siRNA which produced a knockdown of LRP1 but not affected cell's viability. As shown in data presented in Fig. 6, introducing LRP1 siRNA to the cells caused a significant reduction of LRP1 at both mRNA (–53%) and protein (–52%) expression levels as compared to the scrambled siRNA controls (Fig. 6A, B). Confocal microscopic study coupled with laser scanning cytometry further revealed a significant reduction in fluorescent signals corresponding to LRP1 expression in LRP1 knockdown cells as compared to control cells (Fig. 6C, D). Noticeably also, LRP1 knockdown did not visibly alter the confluence or morphology of the cells, suggesting that the siRNA treatment did not produce nonspecific cell death or changes in morphology.

Following optimization of LRP1 siRNA, the Z310 cells were then divided into four groups (Fig. 7): scrambled siRNA control without Pb treatment (Column 1), scrambled siRNA control

with Pb (10 μ M) exposure for 24 h (Column 2), LRP1 knockdown control without Pb treatment (Column 3), and LRP1 knockdown cells with Pb (10 μ M) exposure for 24h (Column 4). A β accumulation was subsequently quantified as before using ELISA. Results revealed a 31% increase ($p < 0.01$) in the Pb exposed group (no LRP1 knockdown) compared to controls as we had seen previously (Column 1 vs. 2 in Fig. 7), thus confirming the finding that Pb exposure increased cellular accumulation of A β . After LRP1 was knocked down and followed by Pb exposure, intracellular A β was further significantly increased in the Pb treated, LRP1 knocked-down cells compared to LRP1 knocked down, no Pb-treated control cells (Column 3 vs. 4 in Fig. 7, $p < 0.001$). Pb exposure in LRP1-knocked down cells led to an accumulation of even more A β than did those control cells with normal LRP1 expression yet with similar Pb exposure (Column 2 vs. 4 in Fig. 7, $p < 0.05$). These results support the hypothesis that increased accumulation in intracellular A β following Pb exposure may be mediated at least in part by Pb effect on LRP1.

Discussion

Our data clearly demonstrate that exposure to Pb results in a significantly increased **accumulation** of A β_{1-40} in rat choroid plexus tissue in vivo and in immortalized choroidal epithelial Z310 cells in vitro. This effect appears to be mediated at least in part, by Pb inhibiting the production of LRP1, a protein implicated in the clearance of A β at the blood brain barrier (Deane et al., 2003; Donahue et al., 2006).

Pb has previously been shown to accumulate in the choroid plexus of humans (Friedheim et al., 1983; Manton et al., 1984) as well as animals (O'Tuama et al., 1976; Zheng et al., 1991; 1996), suggesting that the choroid plexus is a primary target for Pb toxicity following environmental Pb exposure. Our current findings further suggest that upon entering the tissue, Pb ions are not simply sequestered in the choroid plexus, but rather act on critical cellular regulatory mechanisms that mediate A β clearance by this barrier structure.

The effect of Pb on A β accumulation in the choroid plexus shows the following characteristics: (i) occurred in a relatively short time frame, (ii) a dependence on Pb concentration, (iii) somewhat selective to A β regulation in the plexus rather than a consequence of nonselective Pb cytotoxicity, and (iv) a close association with a reduced LRP1 production. A significant increase in A β accumulation in the choroid plexus occurred 24 hr following acute in vivo Pb exposure. Our previous studies demonstrate that under such a dose regimen, the blood Pb concentration reaches above 40 μ g/dL, nearly 4 times above the current reference level of 10 μ g/dL, and Pb concentration in the choroid plexus is about 22 μ g/g of tissue wet weight, nearly 57 fold greater than Pb in brain cortex (Zheng et al., 1991). Remarkably, incubation of Z310 cells with 1–10 μ M of Pb (about 20.7–207 μ g/dL in the culture medium) produced a dose-time dependent increase in cellular accumulation of A β while not causing significant cell damage (i.e., normal cell viability, normal LDH and normal SOD). Hence, it seemed likely and even probable that Pb action on A β accumulation in the choroid plexus may be due to the selective effect of Pb on regulatory processes that mediate A β homeostasis in the choroid plexus.

Several mechanisms may lead to an increased A β level at the blood-CSF barrier: (1) a diminished expulsion of A β molecules from the plexus cells to the extracellular milieu, (2) an increased uptake of A β from the CSF, blood or both, (3) an increased synthesis of A β , and/or (4) a reduced metabolism or degradation of A β . The gene encoding LRP1 contains an Sp1-rich domain in its promoter region (Dawson et al., 1988). Pb is known to alter binding of Sp1 to its targeted DNA sequences (Zawia et al., 2003, 2000). Thus, there was reason to speculate that Pb, by interfering with Sp1 binding capacity, may interfere with gene expression of LRP1. Since extensive studies have revealed a critical role of LRP1 in expelling A β molecules out of the cells at the BBB (Kang et al., 2000; Hyman et al., 2000; Shibata et al., 2000), we sought

to explore the impact of Pb exposure on the LRP1-mediated A β -clearance pathway at the BCB. The data presented in this report indicated that exposure to Pb, either in vivo or in vitro, could lead to a substantial decrease in mRNA and protein levels of LRP1 in the choroid plexus. Conceivably, a decreased LRP1 may result in a functional deficit for the choroidal epithelial cells to expel A β molecules from the cells and therewith an increased cellular accumulation of A β .

To further confirm this point, we conducted LRP1 knockdown experiments using siRNA technique. The results revealed that knocking down LRP1 in conjunction with Pb exposure exacerbated the intracellular accumulation of A β . Hence, it became evident that LRP1 played an important role in transporting A β out of the choroidal epithelial cells, and this process could be altered by Pb exposure. It is noteworthy that LRP levels are known to be significantly reduced in the mid-frontal cortex of AD patients in comparison to those of healthy age-matched control subjects (Kang et al., 2000). Contrary to what we anticipated, the LRP1 knockdown group without Pb exposure did not show an increase in A β accumulation. We speculate that this could be due to the partial knockdown, but not complete knockout, of LRP1 proteins. Also possible is that knocking down LRP1 expression may produce an early compensatory response, leading to a decreased A β uptake in the early stage. Exposure to Pb perhaps increases intracellular A β by a combination of its effect on LRP1 expression, shown in this report, and its action on other mechanisms including alterations in A β uptake. Further studies are thus needed to demonstrate the mechanism.

Our results present a number of interesting questions. First, what is the mechanism by which Pb inhibits the production of LRP1 in the choroid plexus? As mentioned above, the LRP1 gene contains a Sp1 binding domain in one of the three repeat sequences necessary for its transcription (Dawson et al., 1988; Sudhof et al., 1987). Evidence in literature supports a binding of Pb to transcription factor Sp1 (Atkins et al., 2003; Zawia et al., 1998, 2000). Since Sp1 DNA-binding is suggestively regulated by PKC (Atkins et al., 2003) and since Pb can activate PKC (Markovac and Goldstein, 1988; Murakami et al., 1987; Zhao et al., 1998), we postulate that a reduced production of LRP1 following Pb exposure may be the direct and/or indirect result of Pb interacting with PKC-Sp1-DNA regulatory pathway. Research to explore this pathway is currently in progress in this laboratory.

Second, is LRP1 the only pathway affected by Pb exposure? We should point out that this study does not imply nor is apt for the theory that LRP1 is the single most important factor in A β regulation at the BCB. Other mechanisms, particularly receptor-mediated A β uptake and clearance in the choroid plexus, must be considered. Uniquely, the choroidal epithelial cells come into contact with two entirely different body fluid compartments, i.e., the CSF and blood. The presence of A β in both fluid compartments has been established (Chong et al., 2007; Mehta et al., 2000). Thus, for future studies, it is important to investigate whether and how Pb exposure may affect A β uptake mechanisms from either interface in the BCB.

Finally, what are the consequences of A β accumulation in the choroid plexus following Pb exposure? Exposure to Pb has been associated with AD-like symptoms including memory deficits and neurodegeneration, both in adult animals (Wang et al., 2007) and in humans (Shih et al., 2007; Stewart et al., 2006). It still remains unknown whether these memory deficits are due to altered A β homeostasis in brain. As the role of the choroid plexus in cleansing A β via the BCB is gradually becoming evident (Crossgrove et al., 2005), it would be of interest to determine the levels of A β in the CSF and brain tissues as well as the ensuing pathogenic plaques in brain, once the choroid plexus's function in cleansing brain A β is compromised following chronic Pb exposure.

In summary, our results suggest an increased A β accumulation in the blood-CSF barrier located in the choroid plexus after acute Pb exposure in intact animals as well as in cultured BCB cells. Pb-induced A β accumulation in the choroid plexus appears to be due to its inhibition of LRP1, a key intracellular A β transport protein in the choroid plexus. Since the choroid plexus is anatomically adjacent to the hippocampus, a region known to be involved in memory deficits in AD patients, the implication of the altered homeostasis of A β in the CSF due to Pb accumulation in the choroid plexus is of interest for future investigation.

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Fig. 1.

Increased accumulation of intracellular Aβ in rat choroid plexus tissues following in vivo acute Pb exposure by confocal study. (A). Choroid plexus tissue from a control rat. (B). Rats received a single ip injection of 27 mg Pb/kg. Twenty-four hr post exposure, FAM-labeled Aβ₁₋₄₀ was infused into brain ventricles for 0.5 min. The plexus tissues were then removed 20 min post infusion for the confocal study. Substantial stains were evident in the cytosol but not in nuclei of choroidal epithelia. The lower panel shows the corresponding transmission image, indicating normal morphology of plexus tissues. (C). Quantification of the fluorescent signals using Laser Scanning Cytometry. Data represent mean ± SD, n=16 (a total of 16 cells per group taken from 4 tissue samples with fluorescence averaged from 4 cells per sample). **: p<0.001 as compared to controls.

Fig. 2. Increased accumulation of Aβ in immortalized Z310 cells following in vitro Pb exposure by confocal study. (A). Control cells. (B). Cells were incubated with 10 uM Pb for 24 hr. (C). Cells were incubated with 10 μM Pb for 48 hr. An increase in intracellular Aβ signals was evident in Pb-exposed cells as compared to controls. The lower panel shows the corresponding transmission image, indicating a normal morphology of Z310 cells.

Fig. 3.

Increases in accumulation of intracellular A β in Z310 cells following Pb exposure as quantified by ELISA. (A). Dose-response study. Cells were treated with Pb at the concentrations indicated for 24 hr. The cells were harvested and homogenates used for ELSIA. The value in parenthesis was excluded from the linear regression analysis. (B). Time-course study. Cells were treated with 10 μ M for 4–24 h, followed by ELISA. Data represent mean $\pm$ SD, $n = 4$ –6 wells per group. *: $p < 0.05$.

Fig. 4. Decreased LRP1 mRNA expression following in vivo or in vitro Pb exposure. (A). Rats received ip injection of either Na-acetate (control) or Pb acetate (27 mg Pb/kg) and tissues were analyzed 24h after Pb exposure. (B). Z310 cells were treated with 10 μ M Pb for 24h and 48 h. The relative mRNA levels of LRP1 and GAPDH were quantified by real-time RT-PCR and expressed as the ratio of LRP1/GAPDH. Data represent mean $\pm$ SD, n=4; *: p<0.05 as compared to control. The data are representative of triplicate experiments.

Fig. 5.

Decreased LRP1 protein expression following in vivo or in vitro Pb exposure by Western blot analysis. (A) and (B): In vivo study. Rats received ip injection of either Na-acetate (control) or Pb acetate (27 mg Pb/kg) and tissues were analyzed 24h after Pb exposure. Data presented in (B) were estimated from the corresponding band densities in (A) and normalized to those of β-actin. (C) and (D): In vitro study. Z310 cells were treated with 10 μM Pb for 24h or 48 h. Data presented in (D) were estimated from the corresponding band densities in (C) and normalized to those of β-actin. Data represent mean ± SD, n=4; p<0.05 compared to controls.

Fig. 6. Optimization of LRP1 siRNA. Z310 cells were cultured with 50 nM siRNA for 48h and LRP1 knockdown was verified by three independent methods. (A). Representative bands from a Western Blot indicating a 52% knockdown in LRP1 protein expression compared to controls. (B). Reduced LRP1 mRNA expression by real-time RT-PCR analysis. There was a 53% knockdown in LRP1 mRNA. (C). Representative image from Laser scanning cytometry analysis. (D). Quantitative fluorescence intensity. LRP1(-) indicates LRP1 knockdown and control indicates scrambled siRNA. There was a 50% reduction in LRP1-related fluorescent signal in the LRP1(-) group compared to controls. No evident difference in cell confluence was observed between the control and LRP1-knockdown groups. Data represent mean $\pm$ SD, n=4-6; *: p<0.05 as compared to controls. The data are representative of triplicate experiments.

Fig. 7.

Quantification of A β concentrations by ELISA. A plus sign (+) for LRP1(-) (knockdown) indicates the cells with LRP1 knockdown; a minus sign (-) for LRP1(-) indicates the control cells with scrambled siRNA. Following recovery from the transfection of LRP1 or scrambled siRNA, cells were incubated with the media in presence of 10 μ M Pb (with a plus sign) or in absence of Pb (with a minus sign) for 24 h, followed by incubation with 2 μ M A β for 1 h. Data represent mean $\pm$ SD, n = 4–6. Bars with different superscripts are significantly different from one another, $p < 0.05$.

Table 1

Cytotoxicity test of Z310 cells following Pb exposure

Pb (μ M)	MTT Cell Viability	LDH	SOD
0	1.00 $\pm$ 0.037	1.00 $\pm$ 0.234	1.00 $\pm$ 0.059
5	0.963 $\pm$ 0.046	0.951 $\pm$ 0.251	-
10	0.944 $\pm$ 0.102	1.172 $\pm$ 0.35	0.967 $\pm$ 0.058
25	0.888 $\pm$ 0.111	1.174 $\pm$ 0.262	-
50	0.768 $\pm$ 0.04 [*]	1.63 $\pm$ 0.312 [*]	-

The cells were treated with 0–50 μ M Pb for 24 hr and tested for cell viability (MTT assay), membrane permeability (LDH assay) and oxidative stress (SOD assay). Data represent means $\pm$ SD, n = 5–8 as a ratio of control.

^{*}p<0.05 as compared to controls.