SYNERGISTIC EFFECTS OF AMYLOID PEPTIDES AND LEAD ON HUMAN NEUROBLASTOMA CELLS

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Abstract: Aggregated amyloid peptides (AP), major components of senile plaques, have been considered to play a very important and crucial role in the development and neuro-pathogenesis of Alzheimer’s disease (AD). In the present \textit{in vitro} study the synergistic effects of Pb\textsuperscript{2+}, a heavy metal, and AP on the human neuroblastoma SH-SY5Y cells were investigated. The cells treated with Pb\textsuperscript{2+} (0.01-10 µM) alone exhibited a significant decrease in viability and IC\textsubscript{50} was 5 µM. A similar decrease in viability was also observed when the cells were exposed to AP, A\textsubscript{β}1-40 (20-120 µM) and A\textsubscript{β}25-35 (2.5-15 µM) for 48 hrs. The IC\textsubscript{50} values were 60 µM and 7.5 µM for A\textsubscript{β}1-40 and A\textsubscript{β}25-35 respectively. To assess the synergistic effects the cells were exposed to IC\textsubscript{50} of both AP and Pb\textsuperscript{2+}, which resulted in further reduction of the viability. The study was extended to determine the lactate dehydrogenase (LDH) release to assess the cytotoxic effects, 8-isoprostane for extent of oxidative damage, COX 1 and 2 for inflammation related changes, p53 protein for DNA damage and protein kinases A and C for signal transduction. The data suggest that the toxic effects of AP were most potent in the presence of Pb\textsuperscript{2+}, resulting in an aggravated clinical pathological condition. This could be attributed to the oxidative stress, inflammation neuronal apoptosis and an alteration in the activities of the signaling enzymes.

Key words: Amyloid peptides, Lead, LDH, Oxidative stress, Inflammation, Neuronal apoptosis, Signaling

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Abbreviations used: AD – Alzheimer’s disease; A\textsubscript{β} – β-amyloid peptide; AP – aggregated amyloid peptides; APP – amyloid precursor protein; COX – cyclooxygenase; LDH – lactate dehydrogenase; Pb\textsuperscript{2+} – lead; PK – protein kinase; ROS – reactive oxygen species
INTRODUCTION

Alzheimer's disease (AD) is an irreversible and progressive neurodegenerative disorder, which ultimately results in progressive loss of cognitive function, dementia and death [1]. It is characterized by loss of neurons, an abundance of intra-neuronal neurofibrillary tangles composed of protein Tau and extracellular deposition of β-amyloid peptide (Aβ) as amyloid plaques [2, 3]. Though Aβ formation has been considered as a pivotal and crucial step in the pathogenesis of AD [4], the mechanism by which Aβ induces neuronal death is still unknown. It has been shown that during the progression of AD, macromolecular oxidative damage and accumulation of reactive oxygen species (ROS) are prevalent. Oxidative stress, which plays an important role in Aβ-mediated neuronal cytotoxicity by triggering or facilitating neuro-degeneration through a wide range of molecular events, eventually leads to neuronal cell loss [5, 6]. Induction of oxidative stress is involved in mechanisms where production of excessive ROS can mediate neuronal apoptosis in Aβ-induced neuronal cell death [7, 8]. Beta-amyloid peptide (Aβ), derived from sequential proteolysis of the amyloid precursor protein (APP) by β-secretase and γ-secretase, has been shown to trigger neurotoxicity, oxidative damage and inflammation [9-12]. Environmental exposure to low levels of lead (Pb²⁺) is known to exert neurotoxic effects resulting in impairment of higher functions of the brain in infants as well as in adult individuals. Continuous exposure to Pb²⁺ leads to growth and mental retardation, intellectual impairment, neurobehavioral changes and hyperactivity [13, 14]. Other pathological effects, such as suppression of cognition, learning and memory functions, are also characteristic features of Pb²⁺ intoxication. Acute high-dose Pb²⁺ intoxication can also cause encephalopathy with coma, convulsions and frequent fatal outcome [15]. Environmental toxins are among the risk factors that may contribute to the development of AD [16]. A number of epidemiological investigations have demonstrated that among heavy metals, Pb²⁺ is considered to be an important possible risk factor for the pathology of AD. Recent studies from our laboratory reported that Pb²⁺ plays a very important role in the generation of oxidative stress, leading to apoptosis, and initiates inflammatory changes [17]. In the present study the synergistic effects of Pb²⁺ and AP were studied.

MATERIALS AND METHODS

Materials

RPMI-1640 medium and OPI were obtained from GIBCO Life Technologies. Fetal bovine serum, penicillin and streptomycin, and phosphate buffered saline were procured from CELLGRO, Mediatech. Aβ peptides (1-40 and 25-35) were procured from Sigma-Aldrich, St Louis, MO, USA. The kits were used for the assays of MTT cell proliferation (ATCC, Manassas, VA, USA), LDH cytotoxicity assay kit, 8-isoprostane EIA kit (Cayman Chemical Company, Ann
Arbor, MI, USA), Human p53 ELISA kit (Abcam Inc, Cambridge, MA, USA), PKA kinase activity and PKC kinase activity kits (Enzo Life Sciences, PA, USA). All other chemicals were of analytical grade and procured from Sigma.

**Cell culture**

Human SH-SY5Y neuroblastoma cells were used in this study. The cells were grown in RPMI-1640 medium supplemented with 10% (v/v) fetal bovine serum, 50 μg/ml penicillin-streptomycin and OPI (150 μg/ml oxaloacetate, 50 μg/ml pyruvate and 0.2 U/ml insulin) in a humidified air/5% CO2 chamber at 37°C. Medium was changed every three days and cells were passed once they reached approximately 80% confluence.

**Aβ preparation**

Stock (0.1 mM) solutions of Aβ1-40 and Aβ25-35 were prepared using sterile deionized water and stored at -20°C. The stocks were diluted to the desired final concentrations in treatment medium prior to use. The combination of Aβ1-40 and Aβ25-35 was also prepared freshly before the initiation of the experiment.

**Exposure of cells to Aβ1-40 and Aβ25-35 and Pb2+**

Human SH-SY5Y neuroblastoma cells were seeded at 2 x 10^4 cells per well in a 96-well plate. The cells were allowed to attach and grow for two days prior to the treatment. Cells were treated with increasing concentrations of Aβ1-40 (0-120 μM) or Aβ25-35 (0-15 μM), or Pb (0-10 μM). The cells were treated with varying concentrations of Pb2+ (0.01-10 μM) in order to determine the IC50 value and the cell viability was determined by MTT reduction assay.

**MTT reduction assay**

Cell viability was assessed by MTT [3-(4,5-dimethyl-2-thiazolyl)-2,5 diphenyl-2H tetrazolium bromide] reduction assay as per the manufacturer’s instructions. In brief, the cells 2 x 10^5 per well were seeded into 96-well culture plates and allowed to attach. The media containing varying concentrations of Aβ1-40, Aβ25-35 and Pb2+ were added to the cells and incubated for 48 hrs. Then 10 μl of MTT reagent was added to the culture and incubated in the dark for four hours at 37°C followed by cell lysis with the addition of 100 μl of detergent reagent. Then the plate was left at room temperature in the dark for 2 hrs and the relative amount of MTT reduction was determined based on the absorbance measured at 570 nm using a plate reader.

**Determination of LDH cytotoxicity**

The cells were seeded in a 96-well plate at a density of 10^4-10^5 cells/well in 120 μl of culture medium with or without test compounds. The cells were cultured in CO2 incubator at 37°C for 48 hrs and centrifuged at 400 x g for 5 min. 100 μl of the supernatant was transferred into a new plate with 100 μl of reaction solution and the plate was incubated with gentle shaking for about 30 min at room temperature. The absorbance was read at 490 nm using a plate reader.
Determination of 8-isoprostane
To the cells in the non-specific binding wells coated with 8-isoprostane specific rabbit antiserum 50 μl of AChE tracer and 50 μl of 8-isoprostane EIA antiserum were added and the plate was covered with a plastic film and incubated for 18 hrs at 4°C. The wells were emptied and rinsed for 5 times with wash buffer and 200 μl of Ellman’s reagent was added to each well. The plate was covered with plastic film and kept on an orbital shaker for about 120 min and the developed color was read at 420 nm.

COX (1 and 2) activity assay
To the cells in a 96-well plate, 150 μl of assay buffer, 10 μl of heme and 40 μl of sample or standard and 10 μl of inhibitors, DuP-697 (COX-1) or SC-560 (COX-2), were added. The plate was carefully shaken for a few seconds and incubated for 5 min at 25°C. Then 20 μl of colorimetric substrate was added to each well and initiated the reaction by adding 20 μl of arachidonic acid solution. The plate was shaken carefully and incubated for 5 min at 25°C. The absorbance was read at 590 nm using a plate reader.

Determination of p53 levels
In brief, 100 μl of cell lysate/standards were added to the wells of a microtitre plate coated with a monoclonal antibody specific for p53. The plates were washed three times after incubating the plate for 2 hrs at room temperature and 50 μl of biotinylated detection antibody was added. The plates were incubated for 1 hr at room temperature and washed again 3 times. This was followed by the addition of 100 μl of Streptavidin-HRP and incubation for 30 min at room temperature. After adding 100 μl of TMB (tetramethylbenzidine) the plates were kept in the dark for 20 min. To this 100 μl of H2SO4 was added and the absorbance was read at 450 nm using a plate reader.

Determination of protein kinase A (PKA) and protein kinase C (PKC) activity
The PKA substrate pre-coated microtitre plate wells were soaked with 50 μl of kinase assay dilution buffer at room temperature for 10 min. The liquid was carefully aspirated from each well and the samples were added to appropriate wells of the PKA/PKC substrate microtitre plate. The reaction was initiated by adding 10 μl of ATP to each well (except the blank) and incubated for about 90 min at 30°C. The reaction was stopped by emptying the contents of the well. After adding 40 μl of photo-specific substrate antibody to each well and incubating at room temperature for 60 min, the wells were washed four times with 100 μl of wash buffer. Further, 40 μl of diluted anti-rabbit IgG:HRP conjugate was added to each well (except the blank well) and incubated at room temperature for 30 min. The wells were washed with 100 μl of wash buffer followed by the addition of 60 μl of TMB substrate to each well. The plates were incubated at room temperature for about 45 min. The reaction was stopped with 20 μl of stop solution and the absorbance was measured at 450 nm.
Data analysis
Data were expressed as mean ± SD of at least four determinations from each group, repeated at least three times on different occasions. Statistical analysis was performed using one-way analysis of variance (ANOVA) and statistical significance was assumed at p < 0.05.

RESULTS
Human SH-SY5Y neuroblastoma cells were exposed to AP (Aβ1-40 and/or Aβ25-35) for 48 hrs. The viability of cells was significantly reduced from 70.8% to 8.8% when exposed to different concentrations (20-120 μM) of Aβ1-40 (Fig. 1).

Fig. 1. Effect of Aβ (1-40) on human neuroblastoma SH-SY5Y cells. The cells were exposed to increasing (20-120 μM) concentrations of Aβ (1-40) for 48 hrs. The cell viability was determined by MTT cell proliferation assay. Results were expressed as mean ± SD from four samples from each group. *Significantly different from control at p < 0.05.

Fig. 2. Effect of Aβ (25-35) on human neuroblastoma SH-SY5Y cells. The cells were exposed to increasing (2.5-15 μM) concentrations of Aβ (1-40) for 48 hrs. The cell viability was determined by MTT cell proliferation assay. Results were expressed as mean ± SD from four samples from each group. *Significantly different from control at p < 0.05.
A 50% decrease (IC_{50}) in cell viability was observed at 60 μM. The cells exhibited a similar reduction (76.8% to 12.5%) in viability when exposed to different (2.5-15 μM) concentrations of Aβ25-35 (Fig. 2). The IC_{50} was observed at 7.5 μM. A similar concentration-dependent decrease in viability was observed when the cells were exposed to Pb^{2+} (0.01-10 μM) for 48 hrs and the IC_{50} was 5 μM (Fig. 3). Three batches of cells were exposed to IC_{50} of AP or Pb^{2+} or AP + Pb^{2+}. The cell viability was significantly reduced to 54.3%, 50.8% and 44.6% respectively (Fig. 4). LDH release was increased when the cells were

![Graph](image1)

**Fig. 3.** Effect of Pb^{2+} on human neuroblastoma SH-SY5Y cells. The cells were exposed to increasing (0.01-10 μM) concentrations of Pb for 48 hrs. The cell viability was determined by MTT cell proliferation assay. Results were expressed as mean ± SD from four samples from each group. *Significantly different from control at p < 0.05.

![Graph](image2)

**Fig. 4.** Effect of AP + Pb^{2+} on human neuroblastoma SH-SY5Y cells. The cells were exposed to 60 μM Aβ(1-40) + 7.5 μM Aβ(25-35) and 5 μM Pb for 48 hrs. The cell viability was determined by MTT cell proliferation assay. Results were expressed as mean ± SD from four samples from each group. *Significantly different from control at p < 0.05.
exposed to AP (43%) or Pb\(^{2+}\) (37%). The release was further increased (59.4%) when the cells were exposed to both AP and Pb\(^{2+}\) (Fig. 5). A similar increase in the levels of 8-isoprostane was observed when the cells were exposed to AP (47%) or Pb\(^{2+}\) (36%) or AP + Pb\(^{2+}\) (70%) (Fig. 6). The levels of cyclooxygenases (COX-1 and COX-2) were determined in the cells exposed to AP or Pb\(^{2+}\) or AP + Pb\(^{2+}\). The cells exhibited a significant increase in COX-2 but not COX-1 levels. The increase in COX-2 was 59% and 48% in the presence of AP and Pb\(^{2+}\) respectively. The COX-2 levels were further increased to 80% when the cells were exposed to AP + Pb\(^{2+}\) (Fig. 7). A similar increase in the levels of p53 protein

![Fig. 5. Effect of AP + Pb\(^{2+}\) on LDH release in human neuroblastoma SH-SY5Y cells. The cells were exposed to 60 \(\mu\)M A\(\beta\)(1-40) + 7.5 \(\mu\)M A\(\beta\)(25-35) and 5 \(\mu\)M Pb\(^{2+}\) for 48 hrs and the percentage of LDH release was calculated. Results were expressed as mean ± SD from four samples from each group. *Significantly different from control at \(p < 0.05\).](image1)

![Fig. 6. Effect of AP + Pb\(^{2+}\) on 8-isoprostane activity (percentage) in human neuroblastoma SH-SY5Y cells. The cells were exposed to 60 \(\mu\)M A\(\beta\)(1-40) + 7.5 \(\mu\)M A\(\beta\)(25-35) and 5 \(\mu\)M Pb\(^{2+}\) for 48 hrs. Results were expressed as mean ± SD from four samples from each group. *Significantly different from control at \(p < 0.05\).](image2)
was also observed when the cells were exposed to AP (35%) or Pb$^{2+}$ (29%) or AP + Pb$^{2+}$ (45%). The data on PKC and PKA show that the activities of phosphokinases were significantly inhibited in the cells exposed to AP (46% and 36.8%), Pb$^{2+}$ (53% and 42.1%) and AP + Pb$^{2+}$ (33.8% and 31.5%) respectively (Figs. 9 and 10).

Fig. 7. Effect of AP + Pb$^{2+}$ on percentage of COX-1 and COX-2 activity in human neuroblastoma SH-SY5Y cells. The cells were exposed to 60 μM Aβ(1-40) + 7.5 μM Aβ(25-35) and 5μM Pb$^{2+}$ for 48 hrs. Results were expressed as mean ± SD from four samples from each group. *Significantly different from control at p < 0.05.

Fig. 8. Effect of Aβ + Pb$^{2+}$ on p53 levels in human neuroblastoma SH-SY5Y cells. The cells were exposed to 60 μM Aβ (1-40) + 7.5 μM Aβ (25-35) and 5 μM Pb$^{2+}$ for 48 hrs. Results were expressed as mean ± SD from four samples from each group. *Significantly different from control at p < 0.05.
DISCUSSION

Amyloid proteins play an important role in the pathogenesis of AD. Amyloidogenic residues Aβ25-35 and Aβ1-40 have been implicated in neurotoxicity [18, 19]. Neuronal degeneration is said to be a fundamental process responsible for clinical manifestations of many different neurological disorders including AD. Aβ peptides contribute to neuronal and synaptic loss during the course of the disease, resulting in apoptotic cell death [20, 21]. The contribution of environmental factors to the development of AD processes is increasingly becoming more important.

Pb²⁺ remains the number one environmental hazard causing health morbidities in human populations today. The aging populations are vulnerable and have been exposed to much higher levels of environmental Pb²⁺ [22]. Aβ peptides and Pb²⁺ have been shown to cause inflammation, oxidative damage and even the disruption of cell signaling [23-25]. Hence, it is of our interest to study the synergistic effects of two Aβ peptides (1-40 and 25-35) in the presence of Pb²⁺, which is also known to cause similar effects [17]. In the present investigation, we have demonstrated the cytotoxic effect of AP on SH-SY5Y cells using MTT assay, a widely used test for cell viability. The results suggest that AP induce cytotoxicity in a dose- and time-dependent manner. The effect was further enhanced in the presence of Pb²⁺. In support of this, an increase in LDH release was observed in the presence of AP and/or Pb²⁺. LDH, a biomarker, reflects cell membrane integrity, and any damage to the membrane makes the cell release LDH. An increase in LDH release in the presence of AP and/or Pb²⁺ could be attributed to their cytotoxic effects on these cells.
Oxidative stress is induced when ROS production exceeds the capacity of the endogenous antioxidant defense systems. Earlier experimental and human studies reported that oxidative stress is an important causative factor in the development and progression of AD [26, 27]. It has been demonstrated that Aβ-induced neurotoxicity is mediated by free radicals in vitro in a transgenic mouse model of AD [26]. Isoprostanes are a newly discovered group of prostaglandin-like compounds, which are biosynthesized from esterified arachidonic acid through a non-enzymatic free radical-catalyzed mechanism and have very short half-lives [28-30]. Existing evidence advocates that isoprostanes are authentic indicators of free radical-mediated lipid peroxidation and oxidative stress [29, 31, 32]. A significant increase in isoprostane levels in cells exposed to AP and/or Pb suggests that Pb also plays a role in the pathology of AD.

Prostaglandins are synthesized from arachidonic acid by a reaction catalyzed by cyclooxygenase (COX). Earlier studies reported a marked increase in COX-2 expression in cerebral cortex and hippocampus regions of AD brains correlating with the number of senile plaques and neuronal atrophy [33, 34]. It has been shown that AP stimulates the expression of COX-2 and its transcription in neuron cells through a pathway depending on the distal NF-κB response element of the COX-2 promoter [35, 36]. An increase in the COX-2 activity observed in the presence of AP and Pb reflects their involvement in the inflammatory mechanism.

p53 promotes apoptosis and has important implications for the central nervous system, where cell death is observed normally during development in response to injury and in neurodegenerative disorders such as AD [37, 38]. An increase in the expression of p53 in the presence of AP and Pb contributes to induction of oxidative stress and inflammation. Cell proliferation is normally regulated by a complex array of signaling pathways and their integration, which generates a net signaling input that converges on a defined target such as the phosphorylation of transcription factors and proteins that regulate the cell cycle [39, 40]. A significant decrease in the specific activities of both PKA and PKC in the presence of AP and Pb suggests neuronal cell death. The rapid loss of neuronal PKC activity is a common consequence of several forms of brain damage such as ischemia [41, 42]. Decreased levels of the catalytic and regulatory subunits of PKA as well as PKA activity have been observed in the brains of AD patients [43]. In cellular and animal models, Aβ peptides cause accumulation of PKA-R by reducing proteosomal degradation, which leads to a reduction in PKA activity [44-46]. In conclusion, AP and Pb at micromolar concentrations exert neurotoxic effects by inducing oxidative stress and inflammation resulting in apoptosis.

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