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Effects of lead on cholinergic SN56 neuroblastoma cells

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Age-dependent accumulation of lead in brain has been implicated in the pathomechanisms of Alzheimer's disease. The aim of this work was to investigate whether cholinotoxic effects of lead may result from alterations in acetyl-CoA metabolism. One day exposure of differentiated SN56 cholinergic neuroblastoma cells to 0.5 μmol/L lead or 0.01 mmol/L amyloid-β_{1-42} increased fraction of nonviable cells to about 20%. Suppression of choline acetyltransferase activity occurred only in the presence of fresh amyloid-β_{1-42}, whereas lead was ineffective. All agents in combination caused suppression of acetyl-CoA in cytoplasm and mitochondria down to 19% and 34% of controls, respectively. Inverse correlation was observed between whole cell acetyl-CoA level and fraction of nonviable cells at different combinations of lead and other neurotoxic compounds. It indicates that lead had no primary suppressive effect on cholinergic phenotype but, at least in part, exerted cytotoxic influence on cholinergic neurons through the decrease of their acetyl-CoA.

Key words: lead neurotoxicity, acetyl-CoA, choline acetyltransferase, amyloid-β

INTRODUCTION

Environmental exposure to low-levels of lead (Pb) is known to exert neurotoxic effects resulting in an impairment of higher functions of the brain in infants and adult individuals. The suppression of cognition, learning and memory functions were characteristic features of Pb intoxication. Acute, high dose intoxication can cause encephalopathy with coma, convulsions and frequent fatal outcome (Nihei and Guilarte 2002). The lead level in the brain increases with age. It raise the supposition that this toxic heavy metal may be involved in various ageing-related encephalopathies including Alzheimer's disease (AD) (Zawia and Basha 2005). The levels of Pb in blood of humans or in blood and brains of Pb-exposed animals varied from 0.5–3.0 μmol/L with respective variable effects on brain function (Davis et al. 1990, Toscano and Guilarte 2005). Current limit of concern for childhood Pb intoxication, set by Center for Disease Control is 0.5 μmol/L of blood (Toscano and Guilarte 2005).

One of characteristic features of lead overload is impairment of functional and structural integrity of brain cholinergic system in septum and hippocampus of experimental animals (Bielarczyk et al. 1996, Braga et al. 2004). These data are compatible with studies on humans showing the reduction of cholinergic neurons in specific brain areas that correlated with degree of cognitive deficits in AD victims (Auld et al. 1998, Gil-Bea et al. 2005, Schliebs and Arendt 2006). On the other hand, lack of correlation between Aβ load and cognitive deficits in AD may be due to the variable levels of other factors like trace metals, NO or neurotrophins that could either positively or negatively modify the peptide's effects on cholinergic neurons function and viability (Isacson et al. 2002, Bielarczyk et al. 2003, Bishop and Robinson 2004, Zawia and Basha 2005, Hardy 2006). In addition, causes of the

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preferential loss of cholinergic neurons are not clear, but multiple mechanisms are likely to be involved.

It is known that 0.1 μmol/L Pb can inhibit cholinergic inputs through nicotinic receptors in the hippocampus (Braga et al. 2004). The exposure of pregnant rats to Pb in drinking water causes a transient reduction of developmental elevation of choline acetyltransferase (ChAT), high affinity choline uptake and vesicular acetylcholine transporter mRNA in septum and hippocampus of developing but not mature rats (Bielarczyk et al. 1996, Sun et al. 1997). In addition, fetal exposure to Pb was found to cause latent overexpression of amyloid precursor protein in aging brains (Basha et al. 2005a,b). On the other hand, postnatal application of Pb *via* mother's milk to rats resulted in lasting decrease of acetylcholinesterase and increase of acetylcholine contents in their brains (Reddy et al. 2007). Pb is some 100 times stronger than Ca^{2+} agonist in simulating vesicular acetylcholine release and therefore in the presence of nmolar Pb the physiological Ca-dependent release may be impaired (Shao and Suszkiw 1991). An excessive activation of basal ACh release could make the cholinergic neurons more prone to other accompanying toxic signals (Szutowicz 2001). On the other hand, nerve growth factor could prevent cholino-suppressive effects of Pb presumably by independent stimulation of Trk-A-mediated cholino-trophic inputs (Zhou et al. 2000).

There are evidences that Pb may inhibit neuronal energy metabolism (Yun and Hoyer 2000, Yun et al. 2000). The impairment of energy metabolism is also characteristic feature of AD brains. The decreases of pyruvate and ketoglutarate dehydrogenases activities in affected brain areas, with loss of enzyme subunits protein content were observed. They correlated with degree of cognitive and cholinergic deficits in AD patients (Bubber et al. 2005). It raised a question why these conditions were more harmful for cholinergic neurons than for other types of brain cells.

Our previous data indicate that utilization of pyruvate-derived acetyl-CoA for acetylcholine synthesis may generate relative shortage of this metabolite for energy production, particularly during excessive stimulation of cholinergic neurons by neurotoxic signals (Szutowicz 2001, Szutowicz et al. 2006). Such conditions activate acetylcholine re-synthesis to restore transmitter pool in the neurons.

It is known that metals such as Al and Zn, that accumulate in excess in AD brains, may exert cholinergic neurotoxicity itself or in combination with amyloid-β (Aβ) (Zatta et al. 2003). Our studies revealed that cholinergic toxicity of these metals depended in large part on the disturbances in acetyl-CoA metabolism (Szutowicz et al. 2006, Ronowska et al. 2007). Also Pb was reported inhibiting energy metabolism brain *in vivo* and in pathophysiologically relevant 0.5 μmol/L concentration, as well as PDH and hexokinase activities in rat brain homogenates *in vitro* (Yun and Hoyer, 2000, Yun et al. 2000). There is no data whether and how this metal influences activities of these enzymes in brain cholinergic neurons *in vivo*. Also no data are available on acetyl-CoA distribution in Pb-exposed cells. On the other hand, Pb evoked energy depletion in brains of middle aged rats (Yun et al. 2000). In accord with that, the shortage of acetyl-CoA in the mitochondrial compartment in amyloid-β or NO-exposed cholinergic cells was found to be an important factor leading to their death (Bielarczyk et al. 2006). It has been also suggested that Pb may interact with Aβ increasing its cytotoxicity in course of AD encephalopathy (Zawia and Basha 2005).

Therefore the aim of this work was to investigate whether Pb itself or in combination with Aβ might alter acetyl-CoA distribution in cholinergic SN56 neuroblastoma cells. Thereby, putative correlations between Pb-evoked alterations of acetyl-CoA metabolism and cholinergic neurons injury could be revealed. In addition we used submicromolar concentrations of the metal to nick real pathophysiologic conditions taking place in brains of Pb-exposed humans and animals (Davis et al. 1990, Nihei and Guilarte 2002).

METHODS

Materials

Unless otherwise specified biochemicals including lead acetate in were obtained from Sigma-Aldrich (Poznań, Poland), acetyl-CoA [1-^{14}C-acetyl] 4 mCi/mmol was from Perkin-Elmer (Boston, MA, USA). Cell cultures growth media and components were provided by Gibco Life Technologies (Warsaw, Poland), cell culture disposables derived from Sarstedt (Stare Babice, Poland). Amyloid-$\beta_{1\text{-}42}$ was provided by Bachem (Heidelberg, Germany). Nonaggregated (fresh) Aβ 1.0 mmol/L stock solution was prepared in dimethyl sulfoxide. To obtain aggregated (aged) preparation Aβ was dissolved in sterile buffered saline and kept at 37°C for 96 h.

Cell cultures

SN56.B5.G4 cholinergic murine neuroblastoma cell line was a gift from Dr. J.K. Blusztajn (Boston MA, USA). To obtain increased expression of cholinergic phenotype cells were plated at density of 40 000/cm^2 and subcultured in starting Dulbecco's modified Eagle's medium (DMEM) containing 2 mmol/L L-glutamine without or with 1 mmol/L dibutyryl cAMP (cAMP) and 0.001 mM all-*trans*-retinoic acid as differentiating factors and 10% fetal bovine serum at 37°C in atmosphere 5%CO_2, 95% air. Antibiotics, 50 μg streptomycin, 50 IU penicillin and 0.25 μg amphotericin B per 1 ml were used to prevent infections. Differentiation caused morphological maturation and 3–4-fold increase of ChAT activity and acetylcholine level in the cells (Bielarczyk et al. 2003, Szutowicz et al. 2006). On 48th hour of the starting culture cell number reached level about 75% of their final density. At this point both media were replaced by an experimental DMEM without differentiating factors but containing none or selected neurotoxic/neuroprotective agents, as indicated in the text, and culture was continued for following 24 h. Cells were harvested into 10 ml of ice cold HEPES buffered 0.9% NaCl, washed twice by centrifugation at 500 × g for 5 min with some solution and suspended in 320 mmol/L sucrose containing 10 mmol/L HEPES buffer (pH 7.4), 0.1 mmol/L EDTA to obtain the protein concentration about 10.0 mg/ml. Immediately after collection the cells were used for Trypan blue exclusion assay and for metabolic studies. For enzyme assays samples were kept frozen at −20°C for 2–7 days.

Trypan blue exclusion assay

Cell suspension was mixed with equal volume of 0.4% isotonic trypan blue solution. Total cell number and fraction of nonviable, dye accumulating cells were counted after 2 min in Fuchs-Rosenthal haemocytometer under light microscope (Wang et al. 2001).

Metabolic studies

For determination of acetyl-CoA content and distribution harvested cells were incubated in medium containing in a final volume of 1.0 ml 2.5 mmol/L pyruvate, 2.5 mmol/L L-malate, 90 mmol/L NaCl, 30 mmol/L KCl, 20 mmol/L NaHEPES (pH 7.4), 1.5 mmol/L Na-phosphate, 0.01 mmol/L choline chloride, 0.015 mmol/L eserine sulfate, 0.02 mmol/L EDTA, 32 mmol/L sucrose and 0.7–1.0 mg of cell protein. Incubation was continued for 30 min at 37°C with shaking at 100 cycles per min. For determination of total acetyl-CoA content, 0.3 ml of incubation medium was centrifuged at 5 000 × g for 2 min. The supernatant was removed and the cell pellet was deproteinized by suspension in a small volume of 5 mM HCl and incubation in a boiling bath for 1 min. To assess acetyl-CoA content in the cell mitochondria 0.5 ml of the incubation medium was mixed with equal volume of ice cold lysing solution containing 1.4 mg digitonin/ml in 125 mmol/L KCl with 20 mmol/L NaHEPES buffer (pH 7.4) and 3 mmol/L EDTA. Lysate was transferred on 0.5 ml of silicone oil mixture (AR 20 and AR200, 1:2). After 30 s the mitochondrial fraction was separated from the soluble one by centrifugation for 40 s at 12 000 × g. After removal of the soluble fraction and silicon oils, the mitochondrial pellet was deproteinized as described above. The reliability of lysis and separation procedure was verified by the determination of citrate synthase and lactate dehydrogenase activities in mitochondrial and cytoplasmic fractions (Bielarczyk et al. 1998).

Acetyl-CoA assay

Deproteinized extracts of whole cells and mitochondria were treated with maleic anhydride solution in ethyl aether for 2 h to remove CoA-SH. A cycling reaction was carried for 60 min in 0.1 ml of medium containing 1.9 mmol/L acetyl phosphate, 1.2 mmol/L oxaloacetate, 0.72 IU phosphotransacetylase and 0.12 IU citrate synthase. The cycling reaction was stopped by heating samples at 95°C for 6 min and the citrate formed was determined (Szutowicz and Bielarczyk 1987). Cytoplasmic acetyl-CoA level was calculated by subtraction of mitochondrial acetyl-CoA from total acetyl-CoA content (Szutowicz and Bielarczyk 1987).

Enzyme assays

Immediately before the assay samples were thawed and diluted to desired protein concentration in 0.2% v/v Triton X-100. ChAT activity was assessed by the radiometric method using [1-^{14}C]acetyl-CoA as a substrate (Fonnum 1975).

PDH assay was performed by determination of acetyl-CoA formation using citrate synthase trapping system (Szutowicz et al. 1981).

Protein assay

Protein was assayed by the method of Bradford (1976) with human immunoglobulin as a standard.

Statistics

Statistical analyses were carried out by one way ANOVA with Bonferroni multiple comparison *post-hoc* test and $P<0.05$, $P<0.01$, or $P<0.001$ were considered as significant.

RESULTS

One day exposure of differentiated cholinergic cells to 0.5 μmol/L lead acetate (Pb) caused loss of cells extensions, disappearance of intercellular connections and intracellular granulations (not shown). Similar disappearance of mature morphology was observed in the presence of 0.01 mmol/L fresh non-aggregated and aggregated $A\beta_{1\text{-}42}$ (see Bielarczyk et al. 2003, 2006).

Cell number and viability

After 72 h culture in control conditions cells reached subconfluent state corresponding to density of 100 000/cm^2. Pb evoked dose dependent increase of trypan blue positive cell fraction from 5 to about 26% and the decrease of cell number by about 30% at 0.001 mmol/L metal concentration (Fig. 1). The increase of Pb concentration to 0.005 mmol/L caused no further alterations in these parameters (Fig. 1). Therefore, in further experiments, 0.5 μmol/L Pb concentration was used that corresponded to critical low-toxic level in brain and blood *in vivo* (Bielarczyk et al. 1996, Nihei and Guilarte 2002, Toscano and Guilarte 2005) and exerted submaximal cytotoxic effects on SN56 cells (Table I and II). *RS*-Lipoic acid (LA) used alone in 0.01 mM concentration altered neither cell growth nor viability of control or Pb-treated cells (Table I and II).

Fresh $A\beta_{1\text{-}42}$ caused dose-dependent increase of trypan blue-positive cell fraction that reached value 19.5% at 0.01 mmol/L pepetide concentration (Fig. 2). Aggregated $A\beta_{1\text{-}42}$ exerted similar cytotoxic effects as a non-aggregated one (Table I). Combined addition of $A\beta_{1\text{-}42}$ and Pb caused further slight elevation of nonviable cell fraction to about 23% (Table I). Neither Pb nor aggregated $A\beta_{1\text{-}42}$, used alone or combination, decreased cell growth (Table I) On the other hand, fresh $A\beta_{1\text{-}42}$ either alone or combined with Pb reduced cell number by 23 and 31%, respectively (Table I). LA prevented $A\beta_{1\text{-}42}$-evoked detrimental effects on SN56 cells (Table I and II).

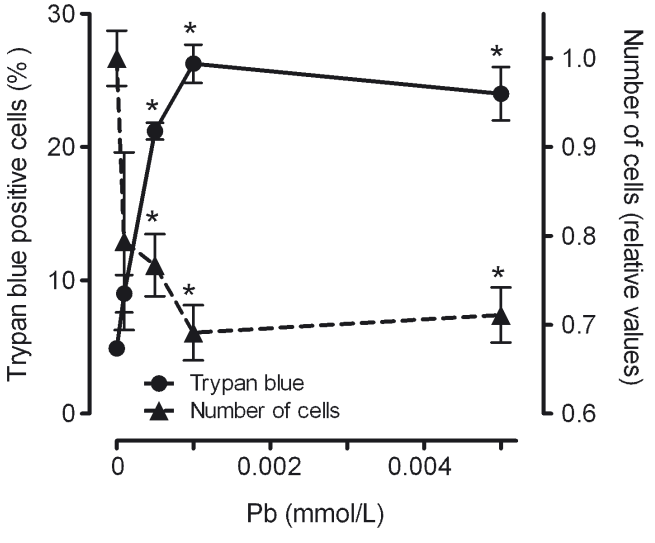

Fig. 1. Dose-dependent effects of lead (Pb) on SN56 cell viability (circles) and their number (triangles). Data are means from 3–6 experiments. Significantly different from: no Pb control *$P<0.01$

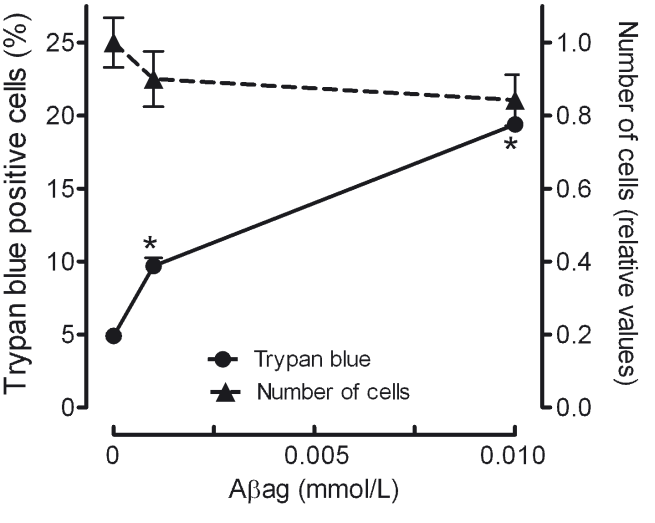

Fig. 2. Dose-dependent effects of aggregated amyloid-$\beta_{1\text{-}42}$ (Aβag) on SN56 cell viability (circles) and their number (triangles). Data are means from 3–6 experiments. Significantly different from: no Pb control *$P<0.01$

Enzyme activities

Exposure of differentiated SN56 cells to fresh $A\beta_{1-42}$ caused 42% inhibition of ChAT activity. Pb neither alone nor in combination with non-aggregated Aβ inhibited ChAT activity (Table II). LA itself slightly suppressed, but simultaneously partially alleviated $A\beta_{1-42}$-evoked reduction of ChAT activity (Table II).

On the contrary, aggregated $A\beta_{1-42}$ alone or in combination with Pb did not alter activity of this enzyme in SN56 cells. Exposure of cells to Pb or Aβ evoked no significant changes in PDH activity.

Acetyl-CoA content

Neither Pb nor fresh or aggregated Aβ alone caused a significant decrease of mitochondrial acetyl-CoA

Table I

Effect of lead and amyloid-β_{1-42} on viability and growth of differentiated cholinergic SN56 neuroblastoma cells

Conditions/ additions (mmol/L)	Trypan blue positive cells (% whole population)	Cell number (10^6/plate)
Control	4.8 ± 0.4	4.35 ± 0.20
Pb 0.0005	20.0 ± 1.2*	3.57 ± 0.16
$A\beta_{1-42}$ fresh 0.01	19.5 ± 2.0*	3.36 ± 0.31
$A\beta_{1-42}$ ag. 0.01	19.4 ± 0.5*	4.27 ± 0.17
Pb 0.0005 + $A\beta_{1-42}$ fresh 0.01	23.1 ± 1.2*	3.01 ± 0.36**
Pb 0.0005 + $A\beta_{1-42}$ ag. 0.01	22.6 ± 1.7*	3.84 ± 0.54
LA 0.01	2.5 ± 0.5	4.44 ± 0.22
Pb 0.0005 + LA 0.01	17.5 ± 1.0*‡	3.20 ± 0.12**‡
$A\beta_{1-42}$ fresh 0.01 + LA 0.01	4.1 ± 0.3†	4.32 ± 0.26

Data are means ± SEM from 6 experiments. Significantly different from: control *P<0.001, **P<0.05; $A\beta_{1-42}$ fresh, †P<0.001; LA alone, ‡ P<0.05

Table II

Effect of lead and amyloid-β_{1-42} on choline acetyltransferase and pyruvate dehydrogenase activities in differentiated cholinergic SN56 neuroblastoma cells

Conditions/ additions (mmol/L)	Choline acetyltransferase nmol/min/mg protein	Pyruvate dehydrogenase nmol/min/mg protein
Control	0.408 ± 0.030	5.26 ± 0.30
Pb 0.0005	0.373 ± 0.049	5.62 ± 0.44
$A\beta_{1-42}$ fresh 0.01	0.262 ± 0.028*	5.79 ± 0.50
$A\beta_{1-42}$ ag. 0.01	0.358 ± 0.058	4.65 ± 0.31
Pb 0.0005 + $A\beta_{1-42}$ fresh 0.01	0.286 ± 0.020*	4.67 ± 0.29
Pb 0.0005+ $A\beta_{1-42}$ ag. 0.01	0.346 ± 0.043	4.41 ± 0.18*
LA 0.01	0.343 ± 0.035	5.97 ± 0.34
Pb 0.0005 + LA 0.01	0.346 ± 0.020	5.85 ± 0.14
$A\beta_{1-42}$ fresh 0.01 + LA 0.01	0.358 ± 0.022†	4.85 ± 0.34

Data are means ± SEM from 6 experiments. Significantly different from: control, *P<0.05, $A\beta_{1-42}$ fresh alone, †P<0.05

(Fig. 3A). However, Pb in combination with fresh or aggregated $A\beta_{1-42}$ depressed acetyl-CoA content in the mitochondria by 65 and 67%, respectively (Fig. 3A). On the other hand, significant 33, 60, and 57% decreases of cytoplasmic acetyl-CoA were observed upon single additions either Pb, or fresh and aggregated $A\beta_{1-42}$, respectively (Fig. 3B). Combined applications of Pb with fresh or aggregated $A\beta_{1-42}$ augmented suppression of cytoplasmic acetyl-CoA level to 30 and 19% of control values, respectively (Fig. 3B). LA itself did not affect compartmentalization but prevented $A\beta_{1-42}$-induced loss of acetyl-CoA in cytoplasmic compartment. However, it was ineffective in Pb-evoked suppression (Fig. 3). Significant inverse correlation (r=0.93, P=0.007) was found between whole cell acetyl-CoA content and cholinergic cell viability in different neurotoxic conditions tested here (Fig. 4).

Analysis of relationships between tested in the past and present neurotoxic effects of different agents and Pb and acetyl-CoA content in SN56 cells also revealed the existence of significant inverse correlation between these parameters (r=0.92, P=0.0002) (Fig. 5) (Jankowska et al. 2000, Bielarczyk et al. 2006, Szutowicz et al. 2006).

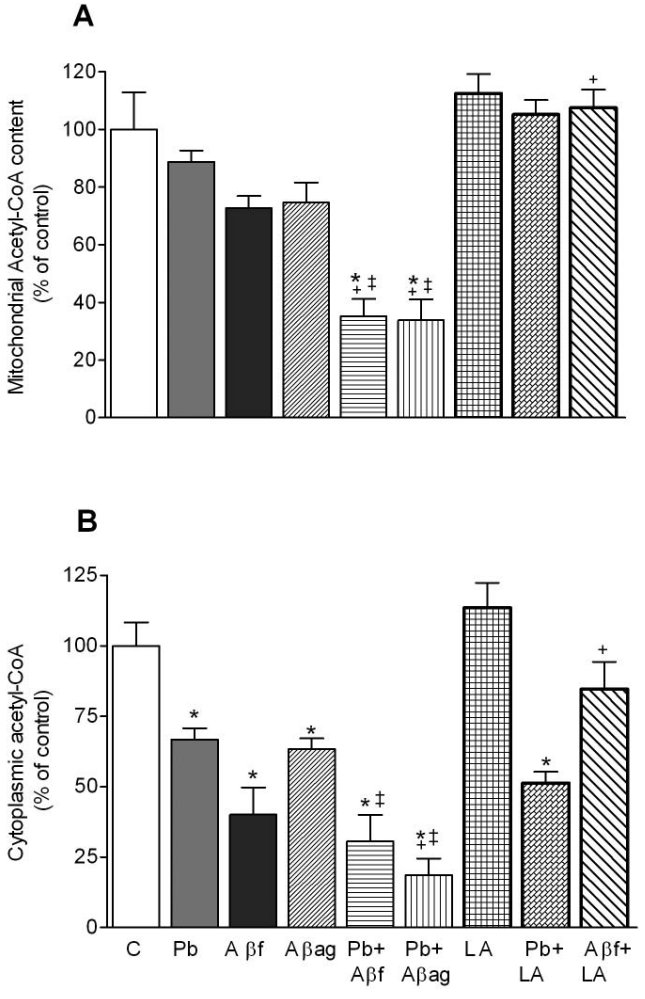

Fig. 3. Effects of 24 hour exposure of SN56 cholinergic differentiated cells to 0.0005 mmol/L lead acetate (Pb) and/or 0.01 mmol/L fresh (Aβf), 0.01 mmol/L aggregated (Aβag) amyloid-β_{1-42} and 0.01 mmol/L lipoic acid (LA) on the level of acetyl-CoA in their mitochondrial (A) and cytoplasmic (B) compartments. Absolute values for acetyl-CoA in controls were: in mitochondria 9.6 ± 1.2, in cytoplasm 37.3 ± 3.1 pmol/mg of whole cell protein. Data are means ± SEM from six experiments. Significantly different from: control; *P<0.05; respective amyloid-β alone, ^{+}P<0.05; Pb alone, ‡P<0.05.

DISCUSSION

It this study we observed that Pb used in 0.5 μmol/L concentration, relevant to its allowable levels in blood and brains of environmentally exposed humans and experimental animals, exerted submaximal neurotoxic effects on cholinergic neuroblastoma cells (Fig. 1,

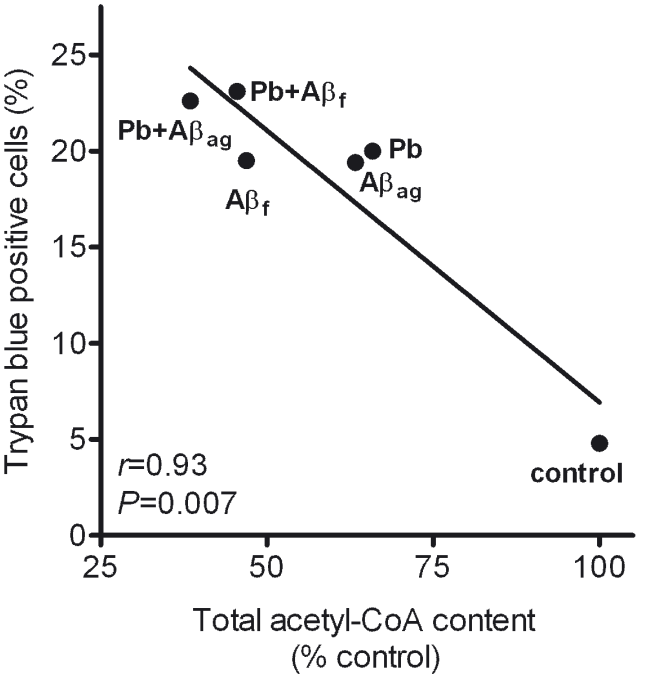

Fig. 4. Inverse correlation between whole cell acetyl-CoA content and trypan blue positive fraction of SN56 cholinergic differentiated cells treated with 0.0005 mmol/L lead acetate (Pb) and/or 0.01 mmol/L fresh (Aβf) and aggregated (Aβag) amyloid-β_{1-42}.

Table I) (Bielarczyk et al. 1996, Yun et al. 2000, Nihei and Guilarte 2002, Toscano and Guilarte 2005). Therefore, the effects of Pb observed here may be of pathophysiological significance for cholinergic neurons in the brain *in vivo*. On the other hand, lack of intensification of neurotoxic effects at Pb concentrations rising above 0.001 mM may be explained by formation of insoluble Pb-phosphate precipitates (Fig. 1).

On the other hand, lack of suppression of ChAT activity despite loss of cell viability in the presence of Pb suggests that metal-dependent mechanism(s) of cell injury might be triggered by this metal much faster than those suppressing cholinergic phenotype (Table I and II). Hence, Pb-evoked death and decomposition of SN56 cells cholinergic cell had occur much faster than expected suppression of cholinergic phenotype (Table I and II). Such discrepancy between cell injury and maintenance of cholinergic phenotype in Pb-treated cells might be also caused by fact that this metal activated cAMP response element binding protein (Zang et al. 2003), which is known to increase the expression of ChAT mRNA (Brock et al. 2007). It could be an independent mechanism compensating Pb-evoked losses in cholinergic functions. However, other studies demonstrated that depression of cell cholinergic phenotype either by aluminum, NO excess or Aβ was paralleled by loss of their viability (Julka et al. 1995, Szutowicz et al. 2006). Lack of inhibitory effect of Pb on ChAT activity in differentiated SN56 neurons (Table II) contrasted also with loss of ChAT activity and ChAT mRNA levels in brain septum of maternally lead-exposed rat pups (Bielarczyk et al. 1996). Reasons for this difference are not clear but may reflect differential susceptibility of various cholinergic cell populations to Pb toxicity or/and longer period of its *in vivo* exposure (Bielarczyk et al. 1996). Indeed, the effects of maternal Pb exposure on ChAT activity in the brain cholinergic neurons of perinatal rat pups were regionally specific (Bielarczyk et al. 1999). Such discrepancy might also result from the fact that, in whole brains of Pb-treated animals the loss of ChAT activity reflected reduction of cholinergic neuron density in respect to noncholinergic cells (Bielarczyk et al. 1996). It was not the case in homogenous cholinergic cell culture where decomposed cells did not contribute to the assessment of specific activity of ChAT in surviving ones (Fig. 1, Table I and II).

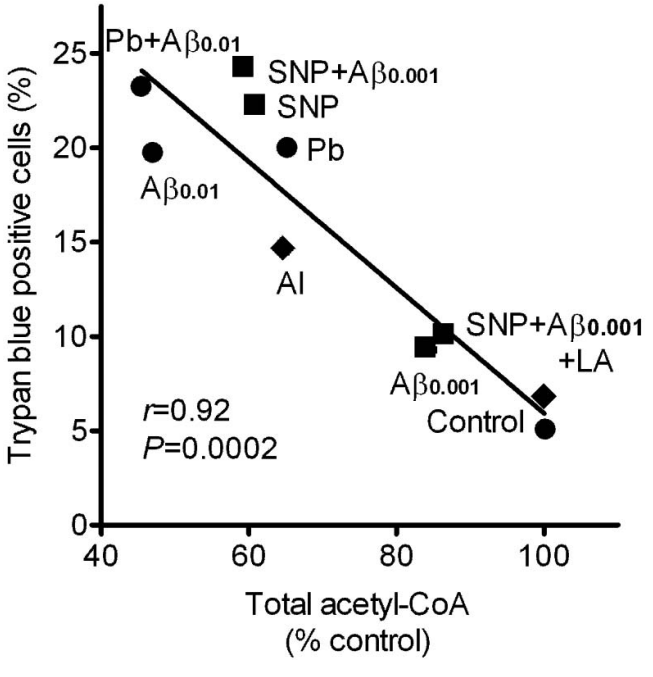

Fig. 5. Combined analysis of inverse correlation between whole cell acetyl-CoA content and trypan blue positive fraction of SN56 cholinergic differentiated cells treated different cytotoxic and cytoprotective factors. Data are from: circles – this paper; squares – Bielarczyk et al. 2006; diamonds – Jankowska et al. 2000, Szutowicz et al. 2006. Abbreviations: (Pb) 0.0005 mmol/L lead acetate; (Aβ) fresh amyloid-β_{1-42} 0.01 or 0.001 mmol/L (as marked); (SNP) 0.4 mmol/L sodium nitroprusside; (Al) 1.0 mmol/L aluminum chloride; (LA) lipoic acid 0.005 mmol/L.

In this study high concentrations both soluble and aggregated Aβ, established AD pathogen, as well as low doses of Pb triggered similar cell damage (Table I). On the contrary, only fresh Aβ suppressed cholinergic phenotype (Table II). Therefore, these data are in line with findings on differential effects of soluble and fibrilar forms of Aβ on cholinergic cells (Heinitz et al. 2006). It is known too, that low nanomolar Aβ concentrations inhibited cholinergic transmission without any cytotoxic effects (Hoshi et al. 1997, Schliebs and Arendt 2006 for review). However, in this report, and in other studies significant toxic effects of Aβ in organotypic and cell culture models appeared at 0.01 mmol/L and higher peptide concentrations (Weiss et al. 1994, Imai et al. 2007, Sirk et al. 2007, Wang et al. 2007). It raises the doubt whether these cytotoxic effects of Aβ were of pathophysiologic significance (Figs 4, 5). However, one may assume that local Aβ concentration in close vicinity of plaques might be much higher than that measured in whole brain.

It has been demonstrated that Pb accelerates Aβ aggregation *in vitro* (Basha et al. 2005b). It raised the supposition that by such mechanism Pb might aggravate Aβ cytotoxicity *in vivo* in the brain (Basha et al. 2005a,b). However, presented data do not support such notion since Pb caused only slight semi-additive augmentation of suppressive effects of both fresh and aggregated Aβ on cell viability (Table I). It may indicate that pathways of suppressive signaling of Pb and Aβ in neuronal cells may be partially super imposable. They could include their interference with Ca and Zn transport and signaling mechanisms (Toscano and Guilarte 2005, Garza et al. 2006). These findings let one to claim that Aβ and Pb may reciprocally aggravate death of cholinergic neurons in course of AD (Table I, Figs 3, 4) (Auld et al. 1998, Zawia and Basha 2005). However, Pb neither itself nor with Aβ would exert a primary chronic cholino-suppressive effects on cell phenotype (Table I and II).

Several agents such as aluminum, sodium nitroprusside or zinc were found to decrease PDH activity and acetyl-CoA level in differentiated cholinergic cells (Bielarczyk et al. 2003, Ronowska et al. 2007). Therefore, toxic effects of these compounds were linked to the decreased synthesis and availability of acetyl-CoA in the mitochondrial compartment. On the other hand, in this and past studies Aβ-evoked suppression of acetyl-CoA and cell viability was accompanied by none or slight drop in PDH activity (Table II) (Bielarczyk et al. 2003). Such discrepancy rises a supposition that this inhibition could occur in cells *in situ* and disappeared in homogenates placed in PDH assay medium (Table II). It is also possible that suppression of cytoplasmic acetyl-CoA, shown here (Fig. 3), could be due to Pb-induced inhibition voltage-gated calcium channels including Ca-activated mitochondrial anionic channel transporting this metabolite to the cytoplasmic compartment (Bielarczyk et al. 1998, Garza et al. 2006). In addition, Pb or Aβ-activated release of acetylcholine could trigger increased utilization of acetyl-CoA for re-synthesis, of depleted transmitter pool (Fig. 3) (Shao and Suszkiw 1991, Szutowicz 2001). However, irrespective of the mechanism, Aβ in combination with Pb, Al or Zn as well as NO excess could deepen acetyl-CoA deficits thereby facilitating functional and structural impairment of cholinergic cells (Figs 1–5) (Zawia and Basha 2005). Significant inverse correlation between cellular acetyl-CoA level and cell impairment at different combinations of neurotoxic agents strongly suggest that Pb and Aβ may reciprocally aggravate their cytotoxic activities through the decrease of acetyl-CoA content in whole cells and their compartments (Figs 3, 4, Tables I and II). These cytotoxic effects of Pb were about 100 times stronger than those observed for Al and Zn (Jankowska et al. 2000, Szutowicz et al. 2006, Ronowska et al. 2007). It indicates that excess each of these metals in combination with accumulated Aβ may reciprocally aggravate cytotoxic effects in AD brains through the decrease of acetyl-CoA in cholinergic neurons (Fig. 3, Table I) (Jankowska et al. 2000, Ronowska et al. 2007, Szutowicz et al. 2006).

LA exerts its neuroprotective activities as cofactor of PDH and ketoglutarate dehdrogenase and as an antioxidant compound (Bielarczyk et al. 2006, Ronowska et al. 2007). Therefore, inability of LA to overcome Pb-evoked cytotoxicity and cholinosuppression indicates that the metal exerted these effects by mechanisms not involving PDH inhibition and free radical generation (Fig. 3, Tables I and II). Fact, that depletion of acetyl-CoA in the cytoplasmic compartment was also insensitive to LA (Fig. 3) raises conclusion that Pb has unique cholinotoxic properties, different from those of other metals involved in AD pathology (Szutowicz 2001, Szutowicz et al. 2006, Ronowska et al. 2007).

Despite of above dissimilarities, presented here data on Pb acetyl-CoA-cell viability relationships (Fig. 3, 4) form a coherent, inverse correlation with data from past studies (Fig. 5) (Jankowska et al. 2000, Bielarczyk et al. 2006, Szutowicz et al. 2006). Thereby they support the hypothesis that alterations in acetyl-CoA level and intracellular distribution may play a substantial role in a lethal response of brain cholinergic neurons to Pb and other multiple neurodegenerative insults, in course of AD.

However, one has to stress that multiple toxic mechanisms may also contribute to Pb-evoked neuronal injuries. They include the inhibition by Pb several classes of voltage-dependent Ca and NMDA channels, impairment of protein kinase C and calcium-calmodulin protein kinase signaling pathways, blockade of cAMP response element binding protein and other transcription factors (Nihei and Guilarte 2002, Toscano and Guilarte 2005, Garza et al. 2006). It remains to be established whether reversible inhibition of acetyl-CoA synthesis in SN56 cells is a primary reaction to Pb exposure or secondary event resulting from disturbances any of pathway(s), described above.

CONCLUSIONS

Presented data revealed the existence of acetyl-CoA-independent cytotoxicity of Pb to cholinergic SN56 neuroblastoma cells, which was not mediated by inhibition of PDH and thereby was insensitive to lipoic acid cytoprotection. On the other hand, suppression of cholinergic phenotype appeared to be sensitive to Pb-evoked depletion of acetyl-CoA in cytoplasmic compartment. Pb could also aggravate acetyl-CoA deficits caused by other AD pathogens. Hence, the impairment of cholinergic neurons in Pb-overloaded brains may result from independent cytotoxic and cholinosuppressive influences of this metal.

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