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Differential toxicity of nitric oxide, aluminum, and amyloid β-peptide in SN56 cholinergic cells from mouse septum

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Abstract

A characteristic feature of several encephalopathies is preferential impairment of cholinergic neurons. Their particular susceptibility to cytotoxic insults may result from the fact that they utilise acetyl-CoA both for energy production and acetylcholine synthesis. In addition, phenotypic modifications of cholinergic neurons are likely to influence their susceptibility to specific harmful conditions. SN56 cholinergic cells were differentiated by the combination of dibutyryl cAMP and retinoic acid. Al and sodium nitroprusside (SNP, NO donor) exerted direct additive inhibitory effects on mitochondrial aconitase activity. However, NO, Al, or amyloid β (Aβ)(25–35) caused none or only slight changes of choline *O*-acetyl transferase (ChAT) and pyruvate dehydrogenase (PDH) activity and relatively small loss of non-differentiated cells (NCs). On the other hand, in differentiated cells (DCs) these neurotoxins brought about marked decreases of these enzyme activities along with greater than in non-differentiated ones increase of cell-death rate. Aβ(35–25) had no effect on these cell parameters. NO and other compounds aggravated detrimental effect of each other particularly in differentiated cells. Thus, differential vulnerability of brain cholinergic neurons to various degenerative signals may result from their phenotype-dependent ratios of acetylcholine to acetyl-CoA synthesising capacities.

Keywords: Cholinergic neurons; NO; Aluminum; β-Amyloid; Choline acetyltransferase; Pyruvate dehydrogenase; Neurotoxicity

1. Introduction

Preferential loss of cholinergic neurons is a characteristic feature of Alzheimer's disease (AD) and several other encephalopathies. Combinations of multiple pathogenic insults including hypoxia and excitotoxic stimulation-evoked increases of cytoplasmic Ca, excessive oxygen and nitrosyl radicals formation, amyloid β (Aβ)-peptide and Al accumulation are involved in mechanisms of neurodegeneration (Greene and Greenamyre, 1996; Torreilles et al., 1999; Meiri et al., 1993). Particular susceptibility of cholinergic neurons to cytotoxic insults may result from the fact that unlike other neurons they utilise acetyl-CoA both for energy production and acetylcholine (ACh) synthesis (Szutowicz et al., 1996). However, this hypothesis needs to be proven.

It is known that NO concentrations increased in brain in course of ischaemia, AD and other degenerative conditions (Torreilles et al., 1999). The excess of NO was found to promote apoptosis in primary neuronal cultures due to peroxynitrite-induced inactivation of respiratory chain complexes, as well as aconitase (aconitate hydratase, EC 4.2.1.3.), pyruvate (pyruvate: lipoate oxidoreductase acceptor acetylating, PDH, EC 1.2.4.1.) and succinate dehydrogenases (succinate: (acceptor) oxidoreductase, EC 1.3.99.1.) (Kim et al., 1999; Brorson et al., 1999; Tomaszewicz et al., 1997). NO was also reported to aggravate Al-evoked elevations of Ca content in cholinergic SN56 cells (Szutowicz et al., 2000). These effects along with those induced by nitrosyl radicals mitochondrial permeability transition state, may explain NO-evoked loss of acetyl-CoA observed in mitochondria of brain synaptosomes and cholinergic SN56 neuroblastoma cells (Balakirev et al., 1997; Szutowicz et al., 1998, 2000; Szutowicz, 2001 for review). In cholinergic neurons acetyl-CoA shortages were likely to be facilitated by NO-evoked several-fold stimulation of ACh release, which triggered its utilisation for re-synthesis of intraneuronal transmitter pool.

Aβ is one of principal pathogens in AD (Selkoe, 1994). Aggregates of Aβ were found to form high conductance Ca channels and activate voltage-dependent ones in cell plasma membranes (Ueda et al., 1997). They also resulted in local

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inflammatory responses of microglial cells that released neurotoxic factors (Combs et al., 2001; Benveniste et al., 2001). In turn, Ca overload was reported to trigger excessive synthesis of oxygen radicals, which impaired acetyl-CoA and energy production (Pereira et al., 1999; Szutowicz et al., 2000; Ueda et al., 1997) due to suppression of PDH, tricarboxylic acid cycle, respiratory chain activity and phosphorylation of Aβ-dependent protein kinases (Hoshi et al., 1997; Keller et al., 1997; Szutowicz et al., 1998). There are indications that cholinergic activity can make neurons more susceptible to Aβ cytotoxicity. For instance RN46A neurons after differentiation to cholinergic phenotype became susceptible, whereas those differentiated to serotoninergic one remained insensitive to Aβ (Olesen et al., 1998). On the other hand, low cholinergic activity was reported to facilitate beta-amyloidogenic proteolytic processing of amyloid precursor protein in brain preparations (Pakaski et al., 2001).

It has been claimed that average brain Al concentrations in some encephalopaties may reach submilimolar levels (Meiri et al., 1993). In agreement with that remain findings, that cultured neuroblastoma cells were capable to accumulate Al against the gradient to milimolar concentrations (Meiri et al., 1993). Despite of that, the significance of aluminum as a primary or secondary pathogenic factor in cholinergic encephalopathies is still a matter of controversy. It has been found, that Al inhibited oxidative metabolism of glucose and pyruvate in brain nerve terminals, presumably due to increased Ca accumulation in their mitochondria (Bielarczyk et al., 1998). This Ca overload was likely to be caused by the inhibition by Al Na/Ca exchanger in the mitochondrial membrane (Szutowicz et al., 1998). Elevated concentration of intramitochondrial Ca could cause excessive release of acetyl-CoA from mitochondria due to increased permeability transition state (Green and Reed, 1998; Szutowicz et al., 1998). Excessive ACh release from Al-treated neurons could in turn increase consumption of cytoplasmic acetyl-CoA for re-synthesis of neurotransmitter pool. Therefore in these conditions the acetyl-CoA depletion took place in cholinergic neuroblastoma cells (Jankowska et al., 2000).

Also several neurotoxic effects of Aβ and NO are mediated through excessive accumulation of Ca in neuronal cells (Szutowicz, 2001 for review). However, there is relatively little information available on how detrimental influences of these factors might combine in course of neurodegenerative processes in the brain. In addition, there is no clear explanation why some groups of brain cholinergic neurons are more and other ones less susceptible to similar neurotoxic conditions. Our hypothesis is that it might depend on the ratio between rates of acetyl-CoA and ACh metabolism in particular cholinergic neurons. Accordingly, cholinergic neurons with low acetyl-CoA synthesising capacity and high expression of cholinergic phenotype would be more prone to neurotoxicity than those with opposite properties (Szutowicz et al., 2000).

In this work we challenged this hypothesis investigating short- and long-term effects of neurotoxic compounds quoted above on viability of non-differentiated and highly differentiated SN56 cholinergic cells. The PDH and choline acetyltransferase (acetyl-CoA: choline *O*-acetyl transferase, EC 2.3.1.6. ChAT) activities were also assayed as indicators of acetyl-CoA and ACh synthesising capacities of these cells under various harmful conditions.

2. Materials and methods

2.1. Materials

Unless otherwise specified biochemicals were provided by Sigma–Aldrich Ltd. (Poznañ, Poland), growth media, and other components were supplied by Gibco-Life Technologies, (Warsaw, Poland), cell culture disposables were obtained from Sarstedt Ltd. (Stare Babice, Poland), Aβ(25–35) and Aβ(35–25) were from Bachem, Heidelberg, Germany; radiochemicals were from Du Pont N.E.N. (Boston, USA), $AlCl_3$ was from Merck (Warsaw, Poland).

Aged Aβ(25–35) and Aβ(35–25) were obtained by incubation of sterile 1.0 mM stock solutions in 0.9% NaCl in 37 °C for 3 days.

2.2. Cell cultures

The SN56.B5.G4 cells created by fusing N18TG2 neuroblastoma cells with murine C57BL/6 neurons from day 21 postnatal day septa, were used (Hammond et al., 1990). Cells were plated at the density of 0.8×10^6 per 6 cm diameter plate and grown for 3 days to subconfluency in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum with 1 mM glutamine and 2.500 IU streptomycin per 1 ml at 37 °C in atmosphere of 95% air and 5% CO_2. Stable differentiation was achieved by culturing cells for 3 days with 0.001 mM all-*trans*-retinoic acid (RA) and/or 1 mM dibutyryl cAMP (Jankowska et al., 2000; Szutowicz et al., 2000; Blusztajn et al., 1992). Differentiated cells (DCs) and non-differentiated cells (NCs) were harvested. Subsequent, experimental passage was performed without differentiating agents in the absence or presence of 1.0 mM $AlCl_3$ and/or 0.001 mM aged Aβ(25–35) or Aβ(35–25). After 24 h culture, 1 mM sodium nitroprusside (SNP) was added for 10 min to selected plates. Then media were removed and all plates were washed with buffered saline. Appropriate culture medium was restored on each plate and incubation continued for the following 48 h. After this time culture media were removed, cells harvested into 10 ml of ice cold 320 mM sucrose containing 10 mM HEPES-buffer (pH 7.4) and 1 mM EDTA, to wash out superficially bound Al, and collected by centrifugation. Such procedure allowed collection only these cells that remained attached to the plate surface. Cells were suspended in a small volume of buffered sucrose without EDTA to make up a protein concentration about 20 mg/ml and used for enzyme assays. Viability of collected cells was assessed by trypan blue exclusion assay

(Spector et al., 1998). Cells that accumulated the dye (trypan blue positive, TBP) were classified as nonviable.

2.3. Enzyme and metabolite assays

Activities of PDH, ChAT and aconitase were assayed after cell disruption with 0.1% Triton X-100 by the methods described elsewhere (Fonnum, 1969; Villafranca, 1974; Szutowicz et al., 1981).

Intracellular ACh was assayed by HPLC method with an enzymatic reactor containing acetylcholinesterase (EC 3.1.1.7.) and choline oxidase (EC 1.1.3.17) with electrochemical detector using commercial kit (Bioanalytical Systems, West Lafayette, USA) with details described elsewhere (Szutowicz et al., 2000). Acetyl-CoA was determined according to Szutowicz and Bielarczyk (1987).

2.4. Western blotting

For studies expression of PDH subunits protein, collected cells were treated with 0.4% Triton X-100, incubated for 15 min in ice and centrifuged 10,000 × *g* for 10 min. Supernatants (50 μg of protein) were subjected to electrophoresis in 9% polyacrylamide gel at 100 V and subsequently transferred to PVDF membrane 0.45 μm (Micron Separations Inc., Westborough, MA, USA) at 130 mA for 12 h. Membrane was blocked 4 h with 3% bovine serum albumin in 150 mM NaCl buffered with 25 mM Tris pH 7.6. Blots were probed with polyclonal rabbit anti-PDH antibody (3.2 μg/ml) (gift from Prof. P.A. Srere) in blocking buffer for 4 h. Membranes were washed five times with buffered saline and treated with goat anti-rabbit IgG antibody conjugated to alkaline phosphatase, diluted 1:20,000 in blocking buffer for 1.5 h. PDH bands were developed in staining reagent containing 0.033% nitro blue tetrazolium and 0.017% 5-bromo-4-chloro-3-indolyl phosphate, 100 mM Tris buffer pH 9, 100 mM NaCl and 5 mM $MgCl_2$. Developing reaction was stopped with 20 mM EDTA. Band density was measured using Bio-Rad Archive Gel Doc Programme (Bio-Rad Laboratories, Munchen, Germany).

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

2.5. Acute effects on Ca content

NCs and DCs grown in toxin free media were used. After collection cells were incubated for 20 min in the absence or presence of 0.25 mM Al or/and 0.20 mM SNP in depolarising medium containing 30 mM KCl and pyruvate as energy substrate, as described earlier (Jankowska et al., 2000). Incubation was terminated by centrifugation for 30 s at 10,000 × *g*. For determinations of whole cell Ca, pellet was suspended in Ca-free medium containing 1 mM EDTA and centrifuged. EDTA was removed by washing pellet with Ca-free medium. For determinations of intramitochondrial Ca, cells membranes were lysed with digitonin and mitochondria separated by centrifugation through silicon oil mixture as described earlier (Bielarczyk and Szutowicz, 1989). Preparations were deproteinised with 5% (w/v) TCA, supernatants were adjusted to pH 7 with NaOH and Ca assayed with arsenazo III (Scarpa, 1979). Extramitochondrial Ca was calculated as a difference between whole cell and intramitochondrial Ca content.

2.6. Statistics

Statistical analyses were carried out by one-way ANOVA with Bonferoni multiple comparison test at $P < 0.05$ being considered to be statistically significant.

3. Results

3.1. Differentiation of SN56 cells

Three day culture of SN56 cells with cAMP and RA resulted in 232% increase of ChAT activity, 202% rise of intracellular ACh content as well as 41% elevation of intracellular Ca along with increased sprouting and formation of synapse-like connections (Table 1). Simultaneously, PDH activity, pyruvate oxidation rate and acetyl-CoA content in DCs were found to be 14, 33 and 30% lower than in NCs, respectively (Table 1). On the other hand, expression of PDH complex subunits, αE1 (pyruvate dehydrogenase, EC 1.2.4.2.), E2 (dihydrolipoamide acetyltransferase, EC 2.3.1.12.) and E3 (dihydrolipoyl dehydrogenase, EC 1.6.4.3.) did not change significantly during differentiation (Fig. 1; Table 2).

Subsequent passages of DCs in growth media devoid of cAMP/RA did not abolish once gained phenotype (Fig. 2).

3.2. Chronic effects of toxins on PDH activity

It is known that activity of PDH determines capacity of neurons for intramitochonrial synthesis of acetyl-CoA

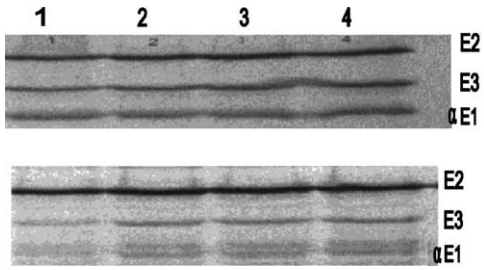

Fig. 1. Effect of Aβ(25–35) and SNP on expression of PDH catalytic subunits in SN56 cells. Representative blots from three independent experiments are shown: (A) non-differentiated; and (B) differentiated. Lanes represent: 1, SNP and Aβ(25–35); 2, SNP; 3, Aβ(25–35); 4, control. E2 data were omitted since no change took place in these experimental conditions.

Table 1
Enzymologic and metabolic parameters in non-differentiated and differentiated SN56 cells

Parameter	Units	Non-differentiated SN56 cells	Differentiated SN56 cells[a]
PDH	nmol/min/mg protein	7.60 ± 0.23	6.58 ± 0.22**
ChAT	nmol/min/mg protein	0.19 ± 0.01	0.63 ± 0.03***
Pyruvate utilisation	nmol/min/mg protein	6.4 ± 0.6	4.3 ± 0.4*
Acetyl-CoA content	nmol/mg protein	31.2 ± 1.5	21.9 ± 1.0***
ACh content	pmol/mg protein	220 ± 37	665 ± 47***
Ca content	nmol/mg protein	29.0 ± 2.6	40.9 ± 3.5*

Data are means ± S.E.M. from 6 to 13 duplicate experiments. Metabolic parameters were assessed after 20 min incubation in Ca-free depolarising medium.
[a] Significantly different from NCs, $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

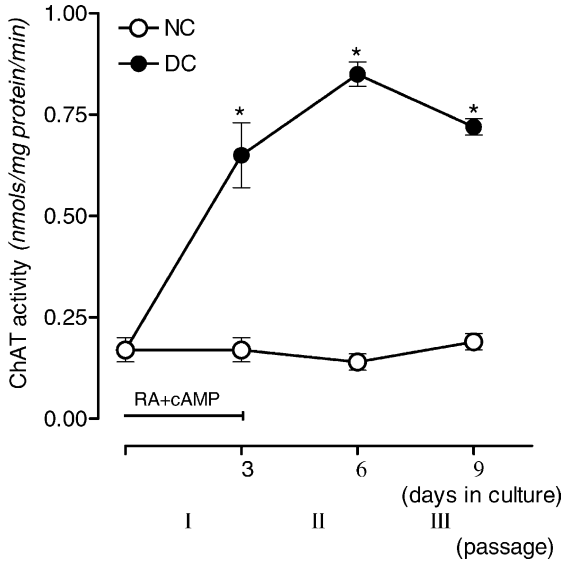

Fig. 2. Stability of cholinergic differentiation of SN56 cells. In passage I 0.001 mM RA and 1.0 mM cAMP were present in the growth medium. They were omitted in passages II and III. Data are means ± S.E.M. from three experiments. Significantly different from NCs, $^{*}P < 0.01$.

(Szutowicz et al., 1996). Neither SNP nor Aβ(25–35) and Al when used alone caused changes in PDH activity in NCs after 3-day culture (Table 3). However in DCs 28, 16 and 39% inhibitory effects of these compounds were observed, respectively. Moreover, when used together they exerted partially additive inhibitory influence on enzyme activity both in NCs and DCs. More distinct effects were found in DCs.

Table 3
Chronic effects of neurotoxins on PDH activity in NCs and DCs

Additions (mM)	Percent of control[a]	
	NCs	DCs
Al 1.0	98.6 ± 8.4	61.0 ± 9.8***
SNP 1.0	92.0 ± 7.6	72.2 ± 2.4**
Aβ(25–35) 0.001	94.9 ± 4.3	84.4 ± 3.7
Aβ(35–25) 0.001	77.1 ± 9.1	105.6 ± 9.5
Al 1.0 + Aβ(25–35) 0.001	38.5 ± 5.6***,#	43.9 ± 5.7***,#
Al 1.0 + Aβ(35–25) 0.001	90.1 ± 6.9	89.5 ± 7.5
SNP 1.0 + Aβ(25–35) 0.001	76.1 ± 3.4*	59.1 ± 4.7***,#
SNP 1.0 + Aβ(35–25) 0.001	107.1 ± 7.0	65.7 ± 7.5**

Data are means ± S.E.M. from five–seven duplicate experiments expressed as a percentage of the respective control NCs and DCs values given in Table 1.
[a] Significantly different from respective control, $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; from Aβ(25–35) or SNP alone $^{\#}P < 0.05$.

On the other hand, equally strong additive effects on PDH activity in both cell groups were observed after joint addition of Aβ(25–35) and Al. None inhibitory effects were brought about by Aβ(35–25) (Table 3).

Decrease of PDH activity in DCs and NCs, evoked by application of any single toxic compound was not accompanied by significant change in expression of any of its three catalytic subunits (Fig. 1; Table 2). However, when Aβ was used with SNP in DCs, 40% suppression of PDH activity was accompanied by about 50% decrease of αE1 and E3 subunits expression (Fig. 1; Tables 2 and 3). On the contrary no such change was found in NCs (Fig. 1; Table 3).

Table 2
Relative intensity units of PDH catalytic subunits expression

Additions (mM)	Relative density units[a]			
	NCs		DCs	
	E3	αE1	E3	αE1
Control	1.62 ± 0.23	1.52 ± 0.20	1.40 ± 0.32	1.37 ± 0.29
Aβ(25–35) 0.001	1.65 ± 0.20	1.45 ± 0.12	1.42 ± 0.21	1.40 ± 0.08
SNP 1.0	1.55 ± 0.18	1.38 ± 0.09	1.40 ± 0.15	1.28 ± 0.20
Aβ(25–35) 0.001 + SNP 1.0	1.60 ± 0.20	1.44 ± 0.16	0.69 ± 0.17*	0.65 ± 0.09*

Data are means ± S.E.M. from three experiments.
[a] Significantly different from control, Aβ(25–35) or SNP alone, $^{*}P < 0.01$.

Table 4
Chronic effects of neurotoxins on ChAT activity in NCs and DCs

Additions (mM)	Percent of control	
	NCs	DCs[a]
Al 1.0	100.0 ± 6.7	70.8 ± 6.2*
SNP 1.0	101.1 ± 5.9	70.3 ± 4.9**
Aβ(25–35) 0.001	111.7 ± 5.3	78.6 ± 7.6
Aβ(35–25) 0.001	110.6 ± 10.3	95.1 ± 3.8
Al 1.0 + Aβ(25–35) 0.001	80.0 ± 5.7#	63.1 ± 6.2***
Al 1.0 + Aβ(35–25) 0.001	101.4 ± 3.2	105.3 ± 10.4
SNP 1.0 + Aβ(25–35) 0.001	85.1 ± 6.4	44.3 ± 6.7***,#
SNP 1.0 + Aβ(35–25) 0.001	96.3 ± 7.0	111.9 ± 6.8

Data are means ± S.E.M. from 5 to 10 duplicate experiments expressed as a percentage of the respective control NCs and DCs values given in Table 1.

[a] Significantly different from respective control, $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; from Aβ or SNP alone $^{\#}P < 0.05$.

3.3. Chronic effects of toxins on ChAT activity

Activity of ChAT is a commonly accepted indicator of differentiation, ACh signalling capacity and structural integrity of cholinergic neurons (Szutowicz et al., 1996). In NCs none of the toxic agents used alone or in combination with the others exerted significant effect on low basal ChAT activity (Tables 1 and 4). On the contrary, in DCs Aβ(25–35), Al and SNP resulted in 21–30% decrease of enzyme activity, respectively (Table 4). When used in combination with Aβ(25–35), they brought about additive inhibitory effect on ChAT activity (Table 3). Note that in DCs ChAT activity was over three times higher than in NCs (Table 1). Aβ(35–25) neither alone nor in combination with Al or SNP affected ChAT activity (Table 4).

3.4. Chronic effects of toxins on cell morphology and viability

None of toxic agents, when used alone, caused appreciable changes in morphology of NCs (Fig. 3) despite the fact that Aβ(25–35) and SNP increased fraction of trypan blue positive cells from 16.2 and 19.6%, whereas Al caused no change in fractional content of nonviable cells, respectively, (Table 5) (Szutowicz et al., 2000). Addition of Aβ(25–35) to SNP-treated NCs caused no further significant increase of TBP but decreased number of polygonal and increased that of round-shaped cells (Fig. 3).

In DCs Al caused no change in TBP but brought about some loss of cell extensions and intercellular connections (Fig. 3; Table 5) (Szutowicz et al., 2000). On the other hand, SNP and Aβ(25–35) caused both significant deterioration of DC morphology and increased number of TBP cells, to the levels which were much higher than in respective NCs (Fig. 3; Table 5). In addition, these deleterious effects appeared to be partially additive. In result, in DCs treated with SNP and Aβ(25–35), fraction of TBP cells was 16% higher than in corresponding NCs (Table 5). No change in number of impaired DCs was seen after application of Aβ(35–25) (Table 5).

Table 5
Chronic effect of neurotoxins on nonviable cell fraction in population of NCs and DCs

Additions (mM)	Trypan blue positive cells (percent of overall population)[a]	
	NCs	DCs
Control	7.0 ± 0.4	8.8 ± 0.4
Al 1.0	7.5 ± 0.4	11.6 ± 2.7
SNP 1.0	20.2 ± 1.2**	27.1 ± 2.3**,***
Aβ(25–35) 0.001	16.3 ± 1.3*	25.8 ± 1.0**,***
Aβ(35–25) 0.001	10.0 ± 0.8	18.1 ± 2.7*
SNP 1.0 + Aβ(25–35) 0.001	22.7 ± 1.1**	36.8 ± 1.1**,***,#
SNP 1.0 + Aβ(35–25) 0.001	16.0 ± 3.1**	19.8 ± 4.0

Results are means ± S.E.M. from 5 to 16 duplicate experiments.

[a] Significantly different from respective control and Al, $^{*}P < 0.01$; $^{**}P < 0.001$; $^{***}P < 0.01$; from respective SNP or Aβ(25–35) alone from respective NCs, $^{\#}P < 0.01$.

3.5. Acute effects of toxins on aconitase activity

It is known that NO and Al exert inhibitory effects on aconitase activity in different preparations of whole brain (Tomaszewicz et al., 1997; Zatta et al., 2000). There is no information on how these compounds may combine their influences on enzyme activity. Pre-incubation of NCs homogenates for 5 min in medium containing 0.2 mM Al or 0.02 mM SNP resulted in inhibition of aconitase activity by 23 and 46%, respectively (Table 7). In same conditions mitochondrial aconitase was depressed by 37 and 58%, respectively. When applied together these agents exerted partially additive effect yielding over 60% inhibition of enzyme activity (Table 7).

3.6. Acute effect of toxins on Ca content

It has been reported that both acute and chronic application of Al or SNP increased Ca content in SN56 cells.

Table 6
Acute effect of Al and SNP on Ca content in non-differentiated SN56 cells

Additions (mM)	Ca^{2+} content (nmol/mg protein)[a]	
	Mitochondria	Cytoplasm
Control	15.4 ± 0.8	16.1 ± 1.5
Al 0.25	25.0 ± 1.8*	17.6 ± 3.9
SNP 0.20	23.3 ± 1.1	14.0 ± 1.4
Al 0.25 + SNP 0.20	30.8 ± 2.6**	14.1 ± 1.8

Data are means ± S.E.M. from four–six duplicate experiments. Ca content was assessed after 20 min incubation in depolarising medium containing 1 mM Ca followed by subcellular fractionation with digitonin solubilisation/silicon oil separation method (Bielarczyk and Szutowicz, 1989).

[a] Significantly different from control, $^{*}P < 0.05$; $^{**}P < 0.001$.

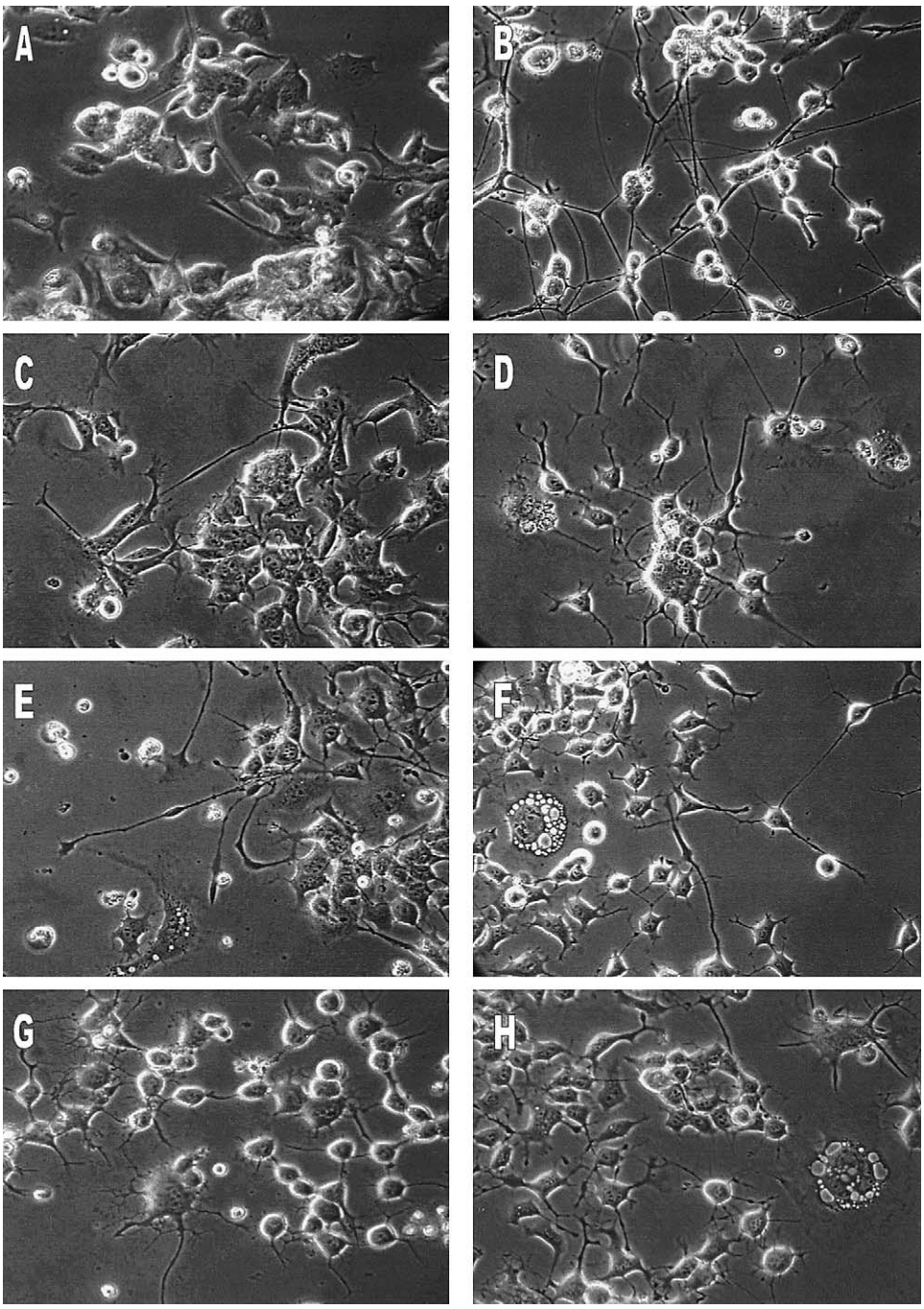

Fig. 3. Representative photomicrographs showing effects of Aβ(25–35) and SNP on SN56 cell morphology. NCs: (A) control; (C) Aβ(25–35); (E) SNP; (G) Aβ(25–35) + SNP. DCs: (B) control; (D) Aβ(25–35); (F) SNP; (H) Aβ(25–35) + SNP.

However, cell compartment(s), in which this accumulation took place was not identified (Jankowska et al., 2000). Addition of 0.25 mM Al or 0.2 mM SNP caused 60 and 50% increase of Ca content in NC mitochondria, respectively. When used together these agents resulted in 100% increase of intramitochondrial Ca (Table 6). Neither SNP nor Al used separately or in combination changed Ca content in cell extramitochondrial compartments.

4. Discussion

Presented data demonstrate that common use of cAMP and RA yielded stable, relatively high differentiation of SN56 cells toward more mature cholinergic phenotype, as demonstrated here by several-fold increases in ChAT activity and ACh content (Fig. 2; Table 1). The DCs also met morphological criteria of partial maturation such as formation

Table 7
Effect of Al and SNP on aconitase activity in non-differentiated SN56 cells

Additions (mM)	Specific activity (nmol/min/mg protein)[a]	
	Homogenate	Mitochondria
Control	40.5 ± 2.5	33.0 ± 2.0
Al 0.2	31.4 ± 1.1**	20.7 ± 1.9**
SNP 0.02	21.9 ± 1.9**	14.0 ± 0.1**
Al 0.5 + SNP 0.02	15.4 ± 3.0**,*	12.2 ± 2.3**,*

Results are means ± S.E.M. from seven (homogenates) and three (mitochondria) experiments. Mitochondria were separated by digitonin solubilisation/silicon oil separation method (Bielarczyk and Szutowicz, 1989).

[a] Significantly different from control, $^{**}P < 0.01$ from Al alone, $^{*}P < 0.05$.

of neural extensions, interneuronal connections and some slow down of cell divisions (Fig. 3A and B) (Szutowicz et al., 2000). This finding remains in accord with earlier data demonstrating that cAMP was capable to evoke stable, differentiation of clonal neuronal cells lasting after subculturing and removal of this differentiating factor (Prasad and Kumar, 1974). Our data provide complementary evidence that also retinoic acid response element in cholinergic gene locus may remain durably activated after subculturing (Fig. 2). This feature of the experimental model enabled one to study chronic effects of neurotoxins on DCs without presence of differentiating agents in the growth medium.

Relatively low PDH activity and pyruvate utilisation in DCs are likely to be caused by inhibition of the enzyme by the excess of Ca accumulated in their mitochondria, since no significant changes were found in enzyme expression. (Fig. 1; Tables 1, 2 and 6) (Lai et al., 1988). Higher Ca level in DCs might result from maturation-evoked increase of Ca-transporting systems of clonal neuronal cells (Gao et al., 1998).

Low rate of pyruvate utilisation in DCs reflects their low acetyl-CoA synthesising capacity. It is demonstrated here by decreased level of this metabolite in DCs in comparison to NC group (Table 1). This finding remains in accord with earlier data demonstrating decrease of acetyl-CoA level in brain preparations after application different inhibitors of PDH (Bielarczyk and Szutowicz, 1989; Gibson et al., 1982).

On the other hand, high activity of ChAT and content of ACh in DCs may reflect their high demand for acetyl-CoA for the transmitter synthesis (Table 1) (Szutowicz et al., 1996). However, causal links between high ChAT activity and size of intracellular pool of ACh remain unknown.

Previous studies have shown that DCs may accumulate significantly higher amounts of Al than NCs (Jankowska et al., 2000). It may explain higher Al-evoked increase of Ca accumulation in DCs as being caused by greater inhibition of Na/Ca exchanger on the mitochondrial membrane (Jankowska et al., 2000; Szutowicz et al., 1998). Excessive accumulation of Ca in DC mitochondria could cause in turn more evident depression of PDH activity (Table 3) and subsequently greater than in NCs decrease of acetyl-CoA content (Jankowska et al., 2000; Lai et al., 1988).

NO-evoked decrease of PDH activity in brain mitochondria was claimed to be caused by its inactivation by nitrosyl radicals (Bolanos and Almeida, 1999; Tomaszewicz et al., 1997). However, if only this possibility would be the case, similar drop of PDH activity should be expected in both experimental groups, what was not the case (Table 3). Therefore, we postulate that in SN56 cells the main cause of PDH inhibition is NO-evoked Ca accumulation in their mitochondria (Table 6) (Lai et al., 1988; Szutowicz et al., 2000). It has been found that in DCs NO caused 80% higher accumulation of Ca than in NCs (Szutowicz et al., 2000). Therefore, observed here differential inhibition of PDH by NO in DCs and NCs may be caused by respective differential rise in mitochondrial Ca concentration (Szutowicz et al., 2000).

Neurotoxic mechanisms of Aβ include formation of high conductance channels for Ca as well as activation of voltage-dependent Ca channels in cell plasma membranes (Ueda et al., 1997). Therefore, Aβ-evoked increase of intramitochondrial Ca is likely to be a factor responsible for inhibition of PDH in DCs (Tables 3 and 6). On the other hand, marked potentiation of Aβ(25–35) inhibitory effect by Al seen in NCs and DCs may be due to facilitation by Al formation of peptide aggregates (Table 3) (Kawahara et al., 2001). Specificity of this and other harmful effects of Aβ(25–35) have been confirmed here by apparent lack of any cytotoxic activity of Aβ(35–25) (Tables 3–5).

Suppression of PDH activity in DCs treated with Aβ(25–35) and SNP may also result from decreased expression of αE1 and E3 enzyme subunits as demonstrated here by Western blot analysis (Fig. 1; Table 2). It may be due to the fact that simultaneous application of these neurotoxins could aggravate rises of Ca and free-radical production to the levels that caused either degradation αE1 and E3 enzyme subunits or/and impairment of their gene expression in the nucleus. This finding demonstrates that single harmful agent could in combination with another one trigger multiple mechanisms leading to impairment of pyruvate utilisation by neuronal cells.

Activity of ChAT in the brain is one of established indicators of integrity of cholinergic neurons. Autopsy studies revealed decrease of ChAT activity in brain cortex of AD victims, which correlated with loss of cholinergic neurons in basal forebrain and the depth of dementia before death (Coyle et al., 1983). Present study demonstrates that depression of cholinergic phenotype in DCs treated with SNP or Aβ(25–35) reflects their damage demonstrated here by respective increase of TBP cells number (Tables 4 and 5). Also loss of differentiated neuron-like morphology by toxin-treated DCs appeared to be concordant with drop of ChAT activity (Table 4, Fig. 3). On the other hand, NCs with low ChAT activity appeared to be much less susceptible to same harmful conditions (Fig. 3; Tables 4 and 5). This particular sensitivity of DCs to neurotoxins may be due to the fact that these conditions resulted in much greater

depression of their PDH activity than in NCs (Table 3). In addition, decreased acetyl-CoA synthesis in DCs was accompanied by simultaneous marked increase of ACh release (Szutowicz et al., 2000). Such conditions are known to stimulate acetyl-CoA utilisation for re-synthesis of intracellular ACh pool. Thereby they may aggravate deficit of acetyl-CoA in highly differentiated cholinergic cells (Szutowicz et al., 1996). On the other hand, NCs with low ChAT activity would better survive in these harmful conditions because they utilised less acetyl-CoA for ACh synthesis (Tables 1 and 3–5).

Inhibition by Aβ cAMP-response element-binding protein signalling was found to be a mechanism leading to depression of ChAT and morphologic differentiation in cortical neurons, which however did not compromise their survival (Tong et al., 2001). Such mechanism did not seem to be a case for SN56 DCs, since Aβ-evoked decrease of ChAT activity was accompanied by respective loss of cell viability (Fig. 3; Tables 4 and 5).

On the other hand, Al suppressed ChAT activity in DCs without affecting their survival, indicating that downregulation of ChAT expression presumably took place in these conditions (Table 4). No direct inhibition of ChAT activity by Al was found (unpublished data). High survival rate of DCs in the presence of Al in growth medium may be due to the fact that Al was added to the medium in high 1 mM concentration which caused inhibition of ACh release (Tables 4 and 5) (Jankowska et al., 2000). Such conditions are known to decrease utilisation of acetyl-CoA for re-synthesis of intracellular neurotransmitter pool (Jankowska et al., 2000). Hence, paradoxic protective effect of Al could be obtained due to suppression of transmitter functions in cholinergic cells.

On the other hand, intracellular Al was found to result in an excessive stimulation of non-quantal ACh release what increased utilisation of acetyl-CoA for re-synthesis of intracellular transmitter pool (Bielarczyk et al., 1998; Jankowska et al., 2000). Thus, the final outcome of Al action on cholinergic neurons could be resultant of their phenotypic properties and extra and intracellular cation concentrations ratio. In our conditions protective and toxic effects of Al were apparently in equilibrium since no change in cell viability was observed (Table 5). These data let one to rise the hypothesis that Al accumulated in brains with Alzheimer's pathology may exert its effect by depressing functional abilities of cholinergic neurons that escaped degeneration. Thereby, Al could aggravate symptoms of already existing cholinergic deficits.

In addition to depression of acetyl-CoA synthesis from pyruvate, NO and Al may also cause inhibition of its utilisation in tricarboxylic acid cycle due to their inhibitory influence on aconitase activity (Table 7) (Tomaszewicz et al., 1997; Zatta et al., 2000). Possible mechanism of inhibitory influence of SNP includes damage by nitrosyl radicals of iron–sulfur complexes in aconitase active centre (Dawson and Dawson, 1995). On the other hand, Al could displace iron from Fe–S clusters due to high stability of its complexes (Table 7) (Harris, 1992). Additive character of Al and NO inhibition suggests that in various brain pathologies detrimental effects of these compounds may combine yielding marked increase of intramitochondrial Ca and severe impairment of both acetyl-CoA provision and its utilisation for energy production (Tables 3, 6 and 7).

This study evidences that otherwise non-toxic concentrations of single neurotoxic compound may in combination with others became harmful to cholinergic neurons. Differential susceptibility of various groups of brain cholinergic neurons to single or combined pathogenic factors may result from the variable expression of their cholinergic and acetyl-CoA metabolism. Presented data remain in accord with other reports showing differential regional susceptibility of cholinergic neurons to NO or Al (Fass et al., 2000; Julka et al., 1995). They are also concordant with finding that cholinergic differentiation of RN46 cells made them more susceptible to Aβ than those differentiated to serotoninergic one (Olesen et al., 1998).

Thus, in highly differentiated cholinergic neurons sustained neurotoxic signals could cause greater ACh release than in less differentiated ones. Such release could trigger in turn greater ACh synthesis, to restore intracellular transmitter pool. If such conditions simultaneously cause inhibition of PDH activity, the shortage of acetyl-CoA and insufficient energy production are likely to occur easier in cholinergic neurons with higher expression of cholinergic functions. Hence, the acetyl-CoA availability in cholinergic neurons may be one of important factors that determine their resistance or susceptibility to pathogenic inputs in course of cholinergic encephalopathies.

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