Free Rad. Res., Vol. 33, pp. 497-506
Reprints available directly from the publisher
Photocopying permitted by license only

Vinpocetine Attenuates the Metabolic Dysfunction Induced by Amyloid β-Peptides in PC12 Cells

CLÁUDIA PEREIRA, PAULA AGOSTINHO and CATARINA R. OLIVEIRA*

Center for Neuroscience of Coimbra, Faculty of Medicine, University of Coimbra, 3004–504 Coimbra, PORTUGAL

Accepted for publication by Prof. H. Sies

(Received 15 October 1999; In revised form 24 March 2000)

The cytoprotective effect of vinpocetine [14-ethoxycarbonyl-(3α,16α-ethyl)-14,15-eburnamine] was investigated on PC12 cells treated with the amyloid β-peptides (Aβ) for 24 hours. Vinpocetine was shown to protect cells from the inhibition in redox status induced by exposure to $A\beta_{25-35}$ and $A\beta_{1-40}$, the maximal protection being achieved at a vinpocetine concentration of 40 μM. At this concentration, vinpocetine blocked the inhibition of the mitochondrial respiratory chain complexes II-III and IV and completely abolished the depletion of pyruvate levels induced by toxic concentrations of Aβ peptides. Furthermore, the accumulation of ROS in cells exposed to $A\beta_{25-35}$ and $A\beta_{1-40}$evaluated using the fluorescent probe 2',7'-dichlorofluorescin (DCF), was reduced in the presence of 40 μM vinpocetine. Taken together, the data presented herein demonstrate that vinpocetine protects cells from Aβ toxicity, preventing the generation of oxidative stress due to the excessive accumulation of ROS. This study suggests that vinpocetine can exert neuroprotective properties which might be of importance and contribute to its clinical efficacy in the treatment of Alzheimer's disease or other neurodegenerative disorders in which oxidative stress is involved.

Keywords: Alzheimer's disease; amyloid β-peptide; energy metabolism; oxidative stress; vinpocetine; antioxidant

INTRODUCTION

Vinpocetine (ethyl-apovincamine-22-oate, Cavinton®), a vincamine synthetic derivative[1], first described as cerebral vasodilator[2], is a widely used nootropic drug exhibiting favourable clinical effects in a variety of dementias[3]. This drug has a neuroprotective effect in a number of experimental models. It has been documented that, *in vivo*, vinpocetine is potent in reducing hypoxic/ischemic brain damage[4–9]. Moreover, vinpocetine exhibits a protective effect against cell death induced by various excitotoxins[10] and inhibits the cytotoxic effect of glutamate in cerebrocortical neurons[11]. Furthermore, several lines of evidence indicate that this drug has significant anticonvulsant potency[12].

Relatively little is known about the underlying molecular mechanisms which might explain these beneficial effects of vinpocetine. It has been reported that vinpocetine possesses some Ca^{2+}-antagonistic activity[13] and inhibits voltage-induced Na^{+}-currents[14,15]. Moreover, the drug was shown to attenuate NMDA responses in oocytes[16], to reduce AMPA binding to corti-

* Corresponding Author: Catarina Resende de Oliveira, Center for Neuroscience of Coimbra, Faculty of Medicine, University of Coimbra, 3004–504 Coimbra, PORTUGAL. Tel.: 351–239–820190; Fax: 351–239–822776; E-mail: croliveira@gemini.ci.uc.pt or cpereira@cnc.uc.pt

cal membranes[17], to inhibit the acetylcholine release evoked by excitatory amino acids[17], and to protect cerebrocortical neurons against excitotoxicity[10]. The potentiation of adenosine-mediated neuroprotection *in vitro*[18] has also been found. Although the mechanism of vinpocetine action is not fully understood, its antioxidant characteristics[11,19] and hydroxyl radical scavenging ability have been demonstrated. Furthermore, VA-045, a novel apovincaminic acid derivative, has been shown to inhibit lipid peroxide production in brain homogenates[20].

As a consequence of the observation that oxidative stress-mediated events can be involved in neurodegenerative disorders, such as Alzheimer's disease (AD), antioxidants have been proposed and used as potential therapeutic agents against oxidative stress-induced neuronal cell death[21,22,23]. Several studies have provided evidence that Aβ, whose abnormal extracellular accumulation in senile plaques constitutes one of the major neuropathological findings in AD[24], can lead to oxidative stress and lipid peroxidation via the induction of H_2O_2 accumulation[25]. We have recently demonstrated that Aβ peptides inhibit the energetic metabolism of PC12 cells, by a mechanism envolving the generation of ROS[26,27]. In the present study, we examined the antioxidant effect of vinpocetine on $A\beta_{25-35}$- and $A\beta_{1-40}$-induced toxicity in rat pheochromocytoma PC12 cells. Cell toxicity due to Aβ peptides was estimated by the determination of the cellular redox status, by the measurement of ROS production and also by the evaluation of the metabolic function (mitochondrial and glycolytic activity).

MATERIALS AND METHODS

Materials

PC12 cells were purchased from ATCC (American Type Culture Collection). Synthetic $A\beta_{25-35}$- and $A\beta_{1-40}$ were purchased from Bachem (Bubendorf, Germany). Vinpocetine was a generous gift from Tecnimed (Portugal). $DCFH_2$-DA was obtained from Molecular Probes (Eugene, OR, USA) and all other reagents from Sigma Chemical Co. (St. Louis, MO, USA). The kit for the determination of pyruvate levels was also obtained from Sigma Chemical Co. (St. Louis, MO, USA).

Toxicity Studies

PC12 cells were incubated, during 24 hours, with the desired concentration of Aβ peptides, $A\beta_{25-35}$ and $A\beta_{1-40}$, or with the corresponding reverse sequences, $A\beta_{35-25}$ and $A\beta_{40-1}$, in RPMI 1640 supplemented with 15% serum. The normal and the reverse sequences of the Aβ peptides were prepared in a similar manner. The $A\beta_{25-35}$ peptide was added from a 1 mg/ml stock prepared in sterile water. The $A\beta_{1-40}$ peptide was also dissolved in sterile water as a 6 mg/ml stock which was then diluted 6 times in PBS and incubated at 37°C for 24 h, in order to induce peptide aggregation. When the protective effect of vinpocetine was tested, cells were treated for approximately 24 h with vinpocetine and then, were simultaneously incubated in the presence of Aβ and the desired concentration of vinpocetine. The vehicle used for the treatment with vinpocetine (HCl 1 N, pH adjusted to 7.4 before use) was also included in control cultures. After an additional 24 h period the cells were analysed.

The evaluation of the cellular redox status was performed by measuring the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction ability of PC12 cells as described previously[28].

Measurement of Intracellular Reactive Oxygen Species Formation

Formation of intracellular ROS was detected using $DCFH_2$-DA[29]. After exposure to $A\beta_{25-35}$ and $A\beta_{1-40}$ (10 nM or 1 μM) for 24 h, in the pres-

ence or in the absence of vinpocetine (40 μM), the cells were incubated in 2 ml of 140 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 5.6 mM glucose, 1.5 mM $CaCl_2$ and 20 mM HEPES-Na, pH 7.4, and allowed to take up 5 μM $DCFH_2$-DA. After loading with $DCFH_2$-DA, at 37°C for 20 min in an atmosphere of 95% air and 5% CO_2, cells were washed with the same buffer and the fluorescence of DCF, the de-esterified and oxidized form of the probe $DCFH_2$-DA, was measured at 502 nm excitation and 550 nm emission, in a SPEX Fluorolog spectrometer.

Activity of Mitochondrial Complexes

All activities were measured in sucrose medium containing (in mM): sucrose 130, KCl 50, $MgCl_2$ 5, KH_2PO_4 5, Hepes 5, pH 7.4.

Complex I (NADH-Ubiquinone oxidoreductase) activity was measured by following the decrease in NADH absorbance at 340 nm, at 30°C, in sucrose medium supplemented with 1 mM KCN, 150 μM NADH, 1.25 mg/ml free fatty acid BSA and PC12 cell homogenates. The reaction was initiated with 50 μM ubiquinone-1, after a preincubation for 5 min[30].

The activity of complex II-III (Succinate dehydrogenase + Succinate-cytochrome c reductase) was determined in sucrose medium supplemented with 8 μM rotenone, 1 mM KCN, 54 μM cytochrome c and cell homogenates. After preincubation for 5 min, the reaction was initiated by addition of 5 mM succinate[31].

The activity of complex IV (Cytochrome oxidase) was measured polarographically at 30°C in homogenates of control or Aβ-treated cells ressuspended in sucrose medium, supplemented with 2 μM rotenone, 0.1 μg antimycin A, 10 μM cytochrome c and 0.3 mg Triton X-100. The reaction was initiated upon addition of 5 mM ascorbate + 0.25 mM *N,N,N′ ,N′*-tetramethyl-*p*-phenylene-diamine (TMPD)[32].

Pyruvate Assay

After incubation with toxic concentrations of $A\beta_{25-35}$ or $A\beta_{1-40}$, PC12 cells were washed twice and scrapped from the dishes with PBS, pH 7.4 at 4°C. Then, an aliquot of the cell suspension was diluted with 8% PCA (1:3, v/v) and proteins were pelleted by centrifugation. The supernatant was stored at −20°C for further spectrophotometric analysis of pyruvate content at 340 nm.

Data Analysis

Data were expressed as means ± S.E.M. of triplicate determinations. Statistical significance analysis was determined using the two-tailed unpaired Student's t test or, for multiple comparisons, the one-way ANOVA, followed by the Tukey-Kramer posthoc test, which compares several groups of values between each other (a value of $p < 0.05$ was considered significant).

RESULTS

Vinpocetine Protects PC12 Cells Against Aβ-Induced Inhibition of Redox Status

The effect of vinpocetine on the inhibition of the cellular redox status induced by Aβ was evaluated by determining the percentage (%) of MTT reduction upon incubation of PC12 cells for 24 h with $A\beta_{25-35}$ or $A\beta_{1-40}$ (10 nM or 1 μM), in the presence of increasing concentrations of vinpocetine. As shown in Fig. 1, vinpocetine, in the concentration range 40–150 μM, induced a significant increase in cellular viability in cells treated with 10 nM and 1 μM of $A\beta_{25-35}$ or $A\beta_{1-40}$. At these concentrations (10 nM and 1 μM), the reverse sequences of the Aβ peptides, $A\beta_{25-35}$ and $A\beta_{1-40}$, were not able to decrease the viability of PC12 cells (Table I).

TABLE I Effect of the reverse sequences of Aβ peptides on the redox status of PC12 cells

Experimental condition	*[Peptide]*	*Redox status (MTT reduction, % of control)*
Control	0	100.1 ± 8.1
$A\beta_{35-25}$ treatment	10 nM	99.3 ± 7.4
	1 μM	101.6 ± 3.3
$A\beta_{40-1}$ treatment	10 nM	112.5 ± 7.8
	1 μM	97.2 ± 2.6

PC12 cells were incubated for 24 h in the absence or in the presence of $A\beta_{35-25}$ or $A\beta_{40-1}$ peptides, 10 nM or 1 μM. The Aβ-induced inhibition of redox status was evaluated by the decrease in the reduction of the tetrazolium salt MTT. The results, expressed as the percentage (%) of control values, are the means ± SEM of triplicate determinations in 6 distinct experiments.

ROS Accumulation Upon Aβ Treatment is Decreased by Vinpocetine

The accumulation of ROS, observed after exposure of PC12 cells to toxic concentrations of $A\beta_{25-35}$ or $A\beta_{1-40}$, was measured in cells treated with these Aβ peptides in the presence or in the absence of 40 μM vinpocetine (Table II). ROS accumulation, estimated utilizing a converting reaction of the probe $DCFH_2$ to DCF, was increased in cells treated with 10 nM or 1 μM $A\beta_{25-35}$ (2.2- and 3.7-fold, respectively) in comparison with controls. $A\beta_{1-40}$ also increased the intracellular levels of ROS at both concentrations tested: 10 nM and 1 μM (2.6- and 4.3-fold, respectively). The increase in DCF fluorescence was significantly attenuated in cells treated during 24 h with the Aβ peptides in the presence of vinpocetine. In cells exposed simultaneously to $A\beta_{25-35}$(10 nM or 1 μM) and 40 μM vinpocetine, ROS accumulation decreased about 40% and in cells pre-incubated with vinpocetine and then exposed to $A\beta_{1-40}$ (10 nM or 1 μM), its accumulation was decreased by approximately 60%.

TABLE II Effect of vinpocetine on Aβ-stimulated intracellular accumulation of peroxides

		ROS accumulation (a.u. DCF fluorescence)	
Experimental condition	*[Aβ peptide]*	*– vinpocetine*	*+ 40 μM vinpocetine*
Control	0	2.02 ± 0.2	1.52 ± 0.1
$A\beta_{25-35}$ treatment	10 nM	4.41 ± 0.4[a]	2.75 ± 0.8[b]
	1 μM	7.54 ± 0.3[c]	4.36 ± 0.5[d]
$A\beta_{1-40}$ treatment	10 nM	5.32 ± 1.1[a]	2.13 ± 0.4[e]
	1 μM	8.63 ± 1.7[c]	2.91 ± 0.5[d]

PC12 cells were incubated with 40 μM vinpocetine. After approximately 24 h, toxic concentrations of $A\beta_{25-35}$ or $A\beta_{1-40}$ peptides (10 nM or 1 μM) were added. The formation of peroxides, detected using $DCFH_2$ oxidation and fluorescence, was assessed 24 h after the addition of the Aβ peptides. The results, expressed as arbitrary units of DCF fluorescence, are the means ± SEM of triplicate determinations in 4 9 distinct experiments.

a. $p < 0.01$, significantly different compared to control conditions, in the absence of $A\beta_{25-35}$ or $A\beta_{1-40}$ peptides.
b. $p < 0.05$, significantly different compared to values determined in $A\beta_{25-35}$ or $A\beta_{1-40}$- treated cells, in the absence of vinpocetine.
c. $p < 0.001$, significantly different compared to control conditions, in the absence of $A\beta_{25-35}$ or $A\beta_{1-40}$ peptides.
d. $p < 0.001$, significantly different compared to values determined in $A\beta_{25-35}$- or $A\beta_{1-40}$- treated cells, in the absence of vinpocetine.
e. $p < 0.01$, significantly different compared to values determined in $A\beta_{25-35}$ or $A\beta_{1-40}$ treated cells, in the absence of vinpocetine.

Vinpocetine Protects PC12 Cells Against Aβ-Induced Inhibition of Complexes II-III and IV of the Mitochondrial Respiratory Chain

After incubation of PC12 cells with toxic concentrations of Aβ, the enzymatic activity of complex I was slightly inhibited (Table III). Its activity decreased approximately 20% in cells treated for 24 h with 1 μM $A\beta_{25-35}$ or $A\beta_{1-40}$, an effect that was not reverted by vinpocetine (40 μM). Similar results were obtained with cells incubated with 10 nM $A\beta_{25-35}$ or $A\beta_{1-40}$.

When the activity of complexes II-III was analyzed, it was observed that it was significantly inhibited by the Aβ peptides (Table IV). Complexes II-III activity decreased approximately 60% in cells treated for 24 h with $A\beta_{25-35}$ or $A\beta_{1-40}$ 1 μM, in comparison with control cells. The inhibition of the activity of complexes II-III was almost completely reverted when cells were incubated with Aβ peptides in the presence of vinpocetine (40 μM). Similar results were obtained with cells incubated with $A\beta_{25-35}$ or $A\beta_{1-40}$ 10 nM.

TABLE III Effect of vinpocetine on the inhibition of mitochondrial respiratory chain complex I activity induced by $A\beta_{25-35}$ or $A\beta_{1-40}$ peptides

		Complex I activity (% of control)	
Experimental condition	*[Aβ peptide]*	*– vinpocetine*	*+ 40 μM vinpocetine*
Control	0	100.0 ± 1.1	100.0 ± 3.5
$A\beta_{25-35}$ treatment	10 nM	78.1 ± 9.2[a]	97.7 ± 9.4
	1 μM	83.7 ± 3.1[a]	83.3 ± 8.1
$A\beta_{1-40}$ treatment	10 nM	81.6 ± 4.5[a]	86.7 ± 7.5
	1 μM	77.4 ± 6.9[a]	81.4 ± 4.8

The enzymatic activity of complex I of control- and Aβ-treated PC12 cells, incubated during 24 h in the presence or in the absence of the vinpocetine (40 μM), was measured as described. Data, expressed as the percentage (%) of control values, are mean ± SEM values of 6–9 different determinations.

a. $p < 0.05$, significantly different compared to control conditions, in the absence of $A\beta_{25-35}$ or $A\beta_{1-40}$ peptides.

TABLE IV Effect of vinpocetine on the inhibition of mitochondrial respiratory chain complex II-III activity induced by $A\beta_{25-35}$ or $A\beta_{1-40}$ peptides

		Complex II-III activity (% of control)	
Experimental condition	*[Aβ peptide]*	*– vinpocetine*	*+ 40 μM vinpocetine*
Control	0	100 ± 1.1	100.0 ± 2.9
$A\beta_{25-35}$ treatment	10 nM	39.7 ± 1.3[a]	80.7 ± 4.3[b]
	1 μM	42.3 ± 5.6[a]	80.0 ± 3.0[b]
$A\beta_{1-40}$ treatment	10 nM	40.1 ± 2.0[a]	79.6 ± 8.4[c]
	1 μM	37.5 ± 4.9[a]	71.8 ± 4.1[b]

The enzymatic activity of complex II-III of control- and Aβ-treated PC12 cells, incubated duting 24 h in the presence or in the absence of the vinpocetine (40 μM), was measured as described. Data, expressed as the percentage (%) of control values, are mean ± SEM values of 6–9 different determinations.

a. $p < 0.001$, significantly different compared to control conditions, in the absence of $A\beta_{25-35}$ or $A\beta_{1-40}$ peptides.
b. $p < 0.001$, significantly different compared to values determined in $A\beta_{25-35}$- or $A\beta_{1-40}$-treated cells, in the absence of vinpocetine.
c. $p < 0.01$, significantly different compared to values determined in $A\beta_{25-35}$ or $A\beta_{1-40}$ treated cells, in the absence of vinpocetine.

TABLE V Effect of vinpocetine on the inhibition of mitochondrial respiratory chain complex IV activity induced by $A\beta_{25-35}$ or $A\beta_{1-40}$ peptides

		Complex IV activity (% of control)	
Experimental condition	*[Aβ peptide]*	*– vinpocetine*	*+ 40 μM vinpocetine*
Control	0	100.0 ± 1.1	100.0 ± 3.4
$A\beta_{25-35}$ treatment	10 nM	33.7 ± 4.5[a]	91.7 ± 4.5[b]
	1 μM	39.8 ± 3.3[a]	89.9 ± 3.0[b]
$A\beta_{1-40}$ treatment	10 nM	45.2 ± 1.1[a]	81.5 ± 8.4[b]
	1 μM	44.0 ± 4.4[a]	82.7 ± 5.4[b]

The enzymatic activity of complex IV of control- and Aβ-treated PC12 cells, incubated duting 24 h in the presence or in the absence of the vinpocetine (40 μM), was measured as described. Data, expressed as the percentage (%) of control values, are mean ± SEM values of 6–9 different determinations.

a. $p < 0.001$, significantly different compared to control conditions, in the absence of $A\beta_{25-35}$ or $A\beta_{1-40}$ peptides.
b. $p < 0.001$, significantly different compared to values determined in $A\beta_{25-35}$- or $A\beta_{1-40}$-treated cells, in the absence of vinpocetine.

TABLE VI Effect of toxic concentrations of $A\beta_{25-35}$or $A\beta_{1-40}$ peptides on endogenous pyruvate levels. Protective action of vinpocetine

		Intracellular pyruvate levels (nmol/mg protein)	
Experimental condition	*[Aβ peptide]*	*– vinpocetine*	*+ 40 μM vinpocetine*
Control	0	8.69 ± 0.4	8.65 ± 0.1
$A\beta_{25-35}$ treatment	10 nM	6.05 ± 1.1[a]	7.69 ± 0.1[b]
	1 μM	5.11 ± 0.1[c]	8.57 ± 0.1[d]
$A\beta_{1-40}$ treatment	10 nM	4.77 ± 0.8[c]	9.77 ± 0.1[e]
	1 μM	5.25 ± 0.5[c]	8.09 ± 0.1[d]

The intracellular pyruvate levels of control- and Aβ-treated PC12 cells, incubated for 24 h in the presence or in the absence of vinpocetine (40 μM), was determined as described. Data are mean ± SEM values of 6–9 different determinations.

a. $p < 0.05$, significantly different compared to control conditions, in the absence of $A\beta_{25-35}$ or $A\beta_{1-40}$ peptides.
b. $p < 0.05$, significantly different compared to values determined in $A\beta_{25-35}$ or $A\beta_{1-40}$ treated cells, in the absence of vinpocetine.
c. $p < 0.001$, significantly different compared to control conditions, in the absence of $A\beta_{25-35}$ or $A\beta_{1-40}$.
d. $p < 0.001$, significantly different compared to values determined in $A\beta_{25-35}$- or $A\beta_{1-40}$-treated cells, in the absence of vinpocetine.
e. $p < 0.01$, significantly different compared to values determined in $A\beta_{25-35}$- or $A\beta_{1-40}$-treated cells, in the absence of vinpocetine.

Complex IV activity was also inhibited by treatment with $A\beta_{25-35}$ or $A\beta_{1-40}$ (Table V). In comparison with controls, its activity decreased approximately 65% in cells treated for 24 h with 1 μM of $A\beta_{25-35}$ and 35% in cells exposed to 1 μM of $A\beta_{1-40}$, respectively. When cells were exposed to toxic concentrations of $A\beta_{25-35}$ or $A\beta_{1-40}$ (1 μM), in the presence of vinpocetine (40 μM), the decrease in the activity of complex IV of the mitochondrial electron transport chain was almost completely abolished (Table V). Similar results were obtained with cells incubated with $A\beta_{25-35}$ or $A\beta_{1-40}$ 10 nM.

Vinpocetine Prevents the Aβ-Induced Depletion of Pyruvate Levels

A significant decline of pyruvate levels was observed after incubation of PC12 cells with 10 nM or 1 μM of $A\beta_{25-35}$ or $A\beta_{1-40}$ (Table VI). Its

levels decreased by 30% and 40% in cells treated with $A\beta_{25-35}$10 nM and 1 μM, respectively. A 45% and 40% decrease was also observed in cells treated, respectively, with $A\beta_{1-40}$ 10 nM or 1 μM. Pyruvate levels, determined in cells treated with $A\beta_{25-35}$ or $A\beta_{1-40}$ in the presence of vinpocetine (40 μM), were similar to those determined in control cells, incubated in the absence of the Aβ peptides (Table VI).

DISCUSSION

The neuroprotective effect of vinpocetine on $A\beta_{25-35}$- or $A\beta_{1-40}$-induced toxicity was investigated in PC12 cells, a valuable model for the analysis of neuronal cell death and neuroprotection. The present study demonstrates that vinpocetine attenuates the toxicity induced by those Aβ peptides in PC12 cells. Vinpocetine, over a concentration range of 40–150 μM, prevented the decrease in cellular redox status (Fig. 1), the accumulation of ROS (Table II) and the inhibition of mitochondrial (Tables III-V) and glycolytic (Table VI) activities, which we have previously shown to occur in PC12 cells upon $A\beta_{25-35}$ or $A\beta_{1-40}$ treatment[26,27].

The protective activity of vinpocetine against Aβ-induced toxicity seems to be due to its free radical scavenging effects because the accumulation of intracellular ROS that follows $A\beta_{25-35}$ or $A\beta_{1-40}$ treatment was prevented in the presence of the drug (Table I). In addition to detect the generation of H_2O_2, $DCFH_2$-DA is also sensitive to peroxynitrite[33]. Since nitric oxide (NO) production has been shown to be stimulated by $A\beta_{25-35}$ [34], and vinpocetine is known to inhibit phosphodiesterase type I, the protective effect of the compound could also be related with a possible interference with the NO and therefore, with the cGMP signaling pathway. The results presented here support previous reports about the antioxidant activity of vinpocetine[11,19]. Its hydroxyl radical scavenging ability has also been demonstrated. Vinpocetine was shown to inhibit lipid peroxidation in brain homogenates and VA-045, a novel apovincaminic acid derivative, is able to inhibit lipid peroxide production[20]. Furthermore, the inhibition of Aβ-induced toxicity was previously shown to be prevented by several antioxidants[21,22], suggesting the involvement of oxidative stress on Aβ-induced toxicity. Moreover, several reports argue in favor of the generation of ROS induced by Aβ fragments[25,35,36]. In recent reports, we have demonstrated that $A\beta_{25-35}$ or $A\beta_{1-40}$ peptides increase ROS accumulation in a dose-dependent manner, this increase being well correlated with the decrease in cell survival, which was inhibited by several antioxidants[27]. However, it can not be ruled out the hypothesis that the protective effect of vinpocetine is due, at least in part, to the inhibition of Aβ aggregation. In fact, a radical generating system has been identified as a Aβ aggregation promoting system and the aggregation could be inhibited by radical scavengers, such as ascorbic acid and vitamin E.

Vinpocetine prevented the $A\beta_{25-35}$- or $A\beta_{1-40}$-induced depletion of pyruvate levels and the inhibition of complexes II-III and IV of the mitochondrial respiratory chain (Tables IV and V). This protective activity of vinpocetine is also consistent with its antioxidant action since we have demonstrated the protective effect of the antioxidants vitamin E, idebenone and GSH-ethyl ester against Aβ-induced depletion of pyruvate levels and inhibition of the mitochondrial complexes I, II-III and IV[27]. On the other hand, the above mentioned neuroprotective activity of vinpocetine may be the consequence of the enhanced cerebral glucose uptake and utilization[9]. These effects might lead to the stimulation of glycolysis and of the mitochondrial electron transport, resulting in the maintenance of pyruvate levels and of the enzymatic activity of complexes of mitochondrial respiratory chain, as we demonstrate in this study.

The protective effect of vinpocetine may also be due to the reduction in Ca^{2+} rise due to Ca^{2+}

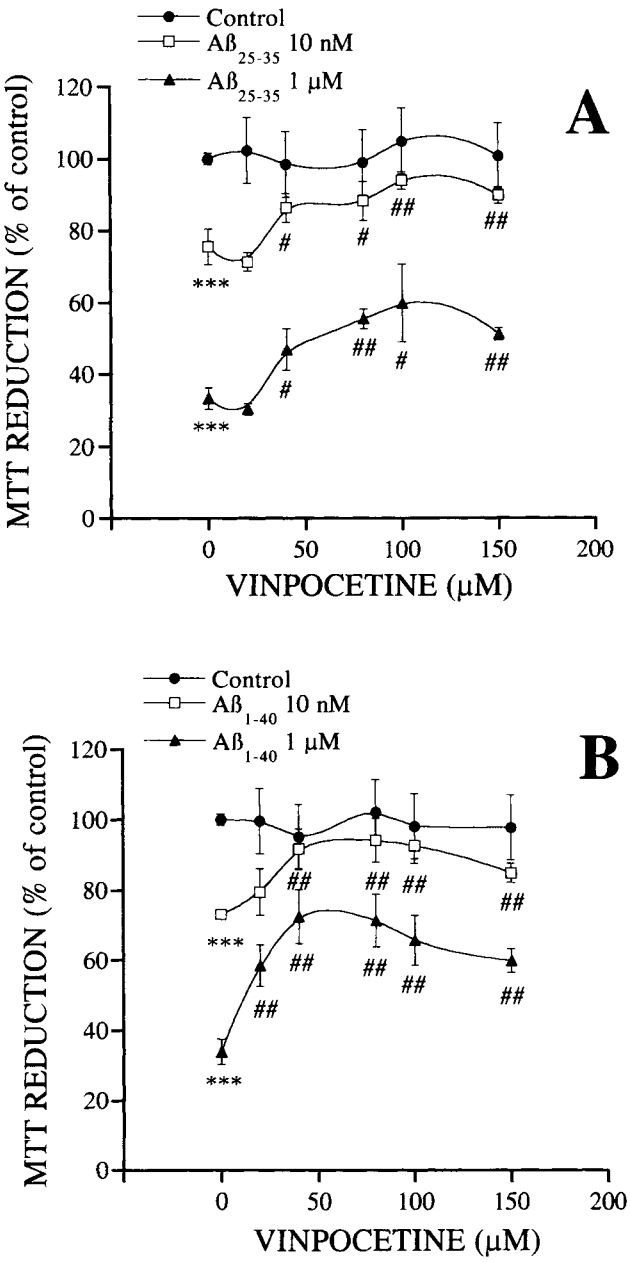

FIGURE 1 Dose-response curves of the neuroprotective effect of vinpocetine against $A\beta_{25-35}$- (A) or $A\beta_{1-40}$- (B) induced inhibition of redox status in PC12 cells. PC12 cells were incubated with increasing concentrations of vinpocetine (20–150 µM) for approximately 24 h. Cells were then incubated for further 24 h in the absence (•) or in the presence of toxic concentrations of $A\beta_{25-35}$ or $A\beta_{1-40}$ peptides, 10 nM (□) or 1 µM (σ). The Aβ-induced inhibition of redox status was evaluated by the decrease in the reduction of the tetrazolium salt MTT. The results, expressed as the percentage (%) of control values, are the means ± SEM of triplicate determinations in 5–10 distinct experiments, respectively. *** $p < 0.001$, significantly different compared to control conditions, in the absence of $A\beta_{25-35}$ or $A\beta_{1-40}$peptides. # $p < 0.05$; ## $p < 0.01$, significantly different compared to values determined in $A\beta_{25-35}$- or $A\beta_{1-40}$-treated cells, in the absence of vinpocetine

influx through voltage-sensitive channels[10,15]. It has been proposed that Aβ exerts its neurotoxic effects through the generation of free radicals that potentiate Ca^{2+} influx through L-type voltage-sensitive Ca^{2+} channels, leading to the increase in $[Ca^{2+}]_i$ and to the colapse of Ca^{2+} homeostasis[37,38,39]. Therefore, the protection afforded by vinpocetine may depend either from its free radical scavenging ability and/or from the inhibition of Ca^{2+} influx through voltage-operated channels and subsequent increase in $[Ca^{2+}]_i$. In fact, it has been shown that, in PC12 cells, Aβ stimulates the influx of Ca^{2+}[40] and increases the $[Ca^{2+}]_i$[41], which can induce the formation of ROS[42].

In conclusion, we have shown for the first time that pretreatment of PC12 cells with vinpocetine can attenuate $A\beta_{25-35}$ or $A\beta_{1-40}$toxicity through a mechanism which seems to be mediated via inhibition of Aβ-induced oxidative stress due to the excessive accumulation of ROS.

Acknowledgements

The present work was supported by FCT (Portuguese Research Council) and the Human Capital Mobility Program (EU), Proposal n°: ERB 4050 PL 932039. Cláudia Pereira is a recipient of grant PRAXIS XXI/BD/3889/94. We are very grateful to Dra. Teresa Proença for her help in pyruvate determinations.

References

[1] Lörinc Cs, Szász K, Kisfaludy L (1976) The synthesis of ethyl apovincaminate. *Arzneimittelforschung* 26, 1907–1913.

[2] E. Kárpáti and L. Szporny (1976) General and cerebral haemodynamic activity of ethyl apovincaminate. *Arzneimittelforschung*, 26, 1908–1912.

[3] R. Balestreri, L. Fontana and F. Astengo (1987) A double-blind placebo controlled evaluation of the safety and efficacy of vinpocetine in the treatment of patients with chronic vascular senile cerebral dysfunction. *Journal of the American Geriatrics Society*, 35, 425–430.

[4] K. Biró, E. Kárpáti and L. Szporny (1976) Protective activity of ethyl apovincaminate on ischemic anoxia of the brain. *Arzneimittelforschung*, 26, 1918–1920.

[5] E. Otomo, J. Atarashi, G. Araki, E. Ito, T. Omal, F. Kuzuya, T. Nukada and O. Ebi (1985) Comparison of vinpocetine with ifenprodil and dihydroergotoxine mesylate treatment and results of long treatment with vinpocetine. *Current Therapeutics Research*, 37, 811–821.

[6] G.A. King (1987) Protective effects ofvinpocetine and structurally related drugs on the lethal consequences of hypoxia in mice. *Archives International Pharmacodynamics*, 286, 299–307.

[7] D. Sauer, R. Rischke, T. Beck, C. Rossberg, H. D. Mennel, G. W. Bielenberg and J. Krieglstein (1988) Vinpocetine prevents ischemic cell damage in rat hippocampus. *Life Sciences*, 43, 1733–1739.

[8] T. Araki, K. Kogure and K. Nishiaka (1990) Comparative neuroprotective effect of pentobarbital, vinpocetine, flunarizine and ifenprodil on ischemic neuronal damage in the gerbil hippocampus. *Research Experimental Medicine*, 190, 19–23.

[9] R. Rischke and J. Krieglstein (1990) Effects of vinpocetine on local cerebral blood flow and glucose utilization seven days after forebrain ischemia in the rat. *Pharmacology*, 41, 153–160.

[10] S. L. Erdö, C. Ning-Sheng, J. R. Wolff and B. Kiss (1990) Vinpocetine protects against excitotoxic cell death in primary cultures of rat cerebral cortex. *European Journal of Pharmacology*, 187, 551–553.

[11] M. Miyamoto, T. H. Murphy, R. L. Schnaar and J. T. Coyle (1989) Antioxidants protect against glutamate-induced cytotoxicity in a neuronal cell line. *Journal of Pharmacology and Experimental Therapeutics*, 250, 1132–1140.

[12] J. Schmidt (1990) Comparative studies on the anticonvulsant effectiveness of nootropic drugs in kindled rats. *Biomedica Biochimica Acta*, 49, 413–419.

[13] S. Kaneko, H. Takahashi and M. Satoh (1990) The use of *Xenopus* oocytes to evaluate drugs affecting brain Ca^{2+} channels: effects of bifemelane and several nootropic agents. *European Journal of Pharmacology*, 189, 51–58.

[14] V. Lakics, M. G. Sebestyén and S. L. Erdö (1995) Vinpocetine is a highly potent neuroprotectant against veratridine-induced cell death in primary cultures of rat cerebral cortex. *Neuroscience Letters*, 185, 127–130.

[15] L. Tretter and V. Adam-Vizi (1998) The neuroprotective drug vinpocetine prevents veratridine-induced $[Na^+]_i$ and $[Ca^{2+}]_i$ rise in synaptosomes. *NeuroReport*, 9, 1849–1853.

[16] S. Kaneko, M. Sugimura, T. Inoue and M. Satoh (1991) Effects of several cerebroprotective drugs on NMDA channel function: evaluation using *Xenopus* oocytes and [^{3}H]MK-801 binding. *European Journal of Pharmacology*, 207, 119–128.

[17] B. Kiss, N. S. Cai and S. L. Erdö (1991) Vinpocetine preferentially antagonises quisqualate/AMPA receptor responses: evidence from release and ligand binding studies. *European Journal of Pharmacology*, 209, 109–112.

[18] J. Krieglstein and R. Rischke (1991) Vinpocetine increases the neuroprotective effect of adenosine in vitro. *European Journal of Pharmacology*, 205, 7–10.

[19] Orviský E, Soltés L, Stančiková M (1997) High-molecular-weight hyaluronan- a valuable tool in testing the antioxidative activity of amphiphilic drugs stobadine and vinpocetine. *Pharmacological and Biomedical Analysis*, 16, 419–424.

[20] Y. Uchiyama-Tsuyuki, S. Okuyama and H. Araki (1996) VA-045, anovel apovincamic acid derivative atteneuates neuronal injury induced by hypoxia or by exci-

tatory amino acids in cultures of rat cortices. *Life Sciences*, 59, 1571–1578.
[21] C. Behl, J. Davis, G. M. Cole and D. Schubert (1992) Vitamin E protects nerve cells from amyloid β protein toxicity. *Biochemical and Biophysical Research Communications*, 186, 944–950.
[22] C. Behl, J. B. Davis, R. Lesley and D. Schubert (1994) Hydrogen peroxide mediates amyloid β protein toxicity. *Cell*, 77, 817–827.
[23] M. Sano, C. Ernesto, R. G. Thomas, M. R. Klauber, K. Schafer, M. Grundman, P. Woodbury, J. Growdon, D. W. Cotman, E. Pfeiffer, L. S. Schneider and L. J. Thal (1997) A controlled trial of selegiline, alpha-tocopherol, or both as treatment for Alzheimers disease. *New England Journal of Medicine*, 336, 1216–1222.
[24] D. J. Selkoe (1993) The molecular pathology of Alzheimer's disease. Neuron, 6, 487–498.
[25] C. Behl, T. Trapp, T. Skutella and F. Holsboer (1997) Protection against oxidative stress-induced neuronal cell death-a novel role for RU486. *European Journal of Neuroscience*, 9, 912–920.
[26] C. Pereira, M. S. Santos and C. R. Oliveira (1998) Mitochondrial function impairment induced by amyloid β-peptide on PC12 cells. *NeuroReport*, 9, 1749–1755.
[27] C. Pereira, M. S. Santos and C. R. Oliveira (1999) Involvement of oxidative stress on the impairment of energy metabolism induced by Aβ-peptides on PC12 cells. Protection by antioxidants. *Neurobiology of Disease*, 6, 209–219.
[28] T. Mosmann (1983) Rapid colorimetric assay for cellular growth and survival. *Journal of Immunological Methods*, 65, 55–63.
[29] J. A. Royall and H. Ischiropoulos (1993) Evaluation of 2′,7′-dichlorofluorescin and dihydrorhodamine 123 as fluorescent probes for intracellular H_2O_2in cultured endothelial cells. *Archives of Biochemistry and Biophysics*, 302, 348–355.
[30] C. I. Ragan, M. T. Wilson, V. M. Darley-Usmar and P. N. Lowe (1987) Subfractionation of mitochondria, and isolation of the proteins of oxidative phosphorylation. In Mitochondria, a Pratical Approach (eds V. M. Darley-Usmar, D. Rickwood and M. T. Wilson), IRL Press, London, pp. 79–112.
[31] H.D. Tisdale (1967) Preparation and properties of succinic-cytochrome c reductase (complex II and III). *Methods in Enzymology*, 10, 213–215.
[32] D. L. Brautigan, S. Ferguson-Miller and E. Margoliash (1978) Mitochondrial cytochrome c: preparation and activity of native and chemically modified cytochrome c. *Methods in Enzymology*, 53, 128–164.
[33] N. W. Kooy, J. A. Royall and H. Ischiropoulos (1997) Oxidation of 2′,7′-dichlorofluorescin by peroxynitrite. *Free Radical Research*, 27, 245–254.
[34] J. Hu and E. E. el-Sakahani (1993) Beta-amyloid activates nitric oxide synthase in a neuronal clone. *NeuroReport*, 6, 760–762.
[35] K. Hensley, J. M. Carney, M. P. Mattson, M. Aksenova, M. Harris, J. F. Wu, R. Foyd and D. A. Butterfield (1994) A model for β-amyloid aggregation and neurotoxicity based on free radical generation by the peptide: relevance to Alzheimer's disease. *Procedings of the National Academy of Sciences of USA*, 91, 3270–3274.
[36] D.A. Butterfield, K. Hensley, M. Harris, M P. Mattson and J. Carney (1994) β-Amyloid peptide free radical fragments initiate synaptosomal lipoperoxidation in a sequence-specific fashion: implications to Alzheimer's disease. *Biochemical and Biophysical Research Communications*, 200, 710–715.
[37] M. P. Mattson, B. Cheng, D. Davis, K. Bryant, I. Lieberburg and R. E. Rydel (1992) β-Amyloid peptides destabilize calcium homeostasis and render human cortical neurons vulnerable to excitotoxicity. *Journal of Neuroscience*, 12, 379–389.
[38] M. P. Mattson, S. W. Barger, B. Cheng, I. Lieberburg, V. L. Smith-Swintosky and R.E. Rydel (1993) β-amyloid precursor protein metabolites and loss of neuronal Ca^{2+} homeostasis in Alzheimer's disease. *Trends in Neuroscience*, 16, 409–414.
[39] Y. Goodman, M. R. Steiner, S. M. Steiner and M. P. Mattson (1994) Nordihydroguaiaretic acid protects hippocampal neurons against amyloid β-peptide toxicity, and attenuates free radical and calcium accumulation. *Brain Research*, 654, 171–176.
[40] R. Fukuyama, K. C. Wadhwani, Z. Galdzicki, S. I. Rapoport and G. Ehrenstein (1994) β-Amyloid polypeptide increases calcium-uptake in PC12 cells: a possible mecahanism for its cellular toxicity in Alzheimer's disease. *Brain Research*, 667, 269–272.
[41] S. Yallampalli, M.-A. Micci and G. Taglialatela (1998) Ascorbic acid prevents β-amyloid-induced intracellular calcium increase and cell death in PC12 cells. *Neuroscience Letters*, 251, 105–108.
[42] T. K. Mcintosh, K. E. Saatman and R. Raghupathi (1997) Calcium and the pathogenesis of traumatic CNS injury: cellular and molecular mechanisms. *The Neuroscientist*, 3, 169–175.