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Comparative Studies on $[Ca^{2+}]_i$-Level of Fibroblasts from Alzheimer Patients and Control Individuals

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The accumulation of the β-amyloid peptide (βAP) in the brain, produced from the ubiquitously expressed amyloid precursor protein (APP) is a defining feature of Alzheimer's disease (AD). Consistent with studies demonstrating the importance of skin biopsy in the diagnosis of neurodegenerative disorders, we investigated whether differences in intracellular free calcium levels ($[Ca^{2+}]_i$) of cultured cutaneous fibroblasts derived from sporadic AD patients and from age-matched control individuals might be present. $[Ca^{2+}]_i$ was measured in Fura-2AM-loaded human fibroblasts by dual wavelength spectrofluorimetry. AD cells exhibited lower $[Ca^{2+}]_i$ as compared to the control cultures. Exposure of fibroblasts to βAP resulted in increased $[Ca^{2+}]_i$ of the control cells, but not of AD fibroblasts. Our test could prove useful in supporting the diagnosis of (sporadic) AD in patients suspected of suffering from the disease.

KEY WORDS: Alzheimer's disease; β-amyloid; fibroblast; fluorescence.

INTRODUCTION

Senile plaques and neurofibrillary tangles comprise the major neuropathological lesions of Alzheimer's disease (AD). The plaques contain extracellular deposits of β-amyloid (βAP) protein, which form abundant amyloid fibrils (7–10nm) intermixed with amorphous aggregates of this peptide. Deposits are composed of $βAP_{42}$, the 42 amino-acid-residue form of the peptide. Chronic elevation of $βAP_{42}$ in brain interstitial fluid (and also inside neurons) gradually leads to oligomerization and fibrillation of the peptide. Accumulation of $βAP_{42}$ initiates inflammatory and neurotoxic cascades. Progressive neuritic injury results in disruption of neural metabolic and ionic homeostasis. Destabilization of Ca^{2+} homeostasis in neurons plays a central role in AD pathogenesis (1).

Exposure to βAP elicits sustained activation of Ca^{2+}-permeable receptor channels, which results in a pathological enhancement of inward Ca^{2+} currents and a subsequent increase in the $[Ca^{2+}]_i$ (2–5). Elevation of $[Ca^{2+}]_i$ may further progress through the activation of second messengers that mobilize intracellular Ca^{2+} (e.g., IP_3) resulting in Ca^{2+} release from its intracellular stores (6–7). As a result, $[Ca^{2+}]_i$ may reach pathological concentrations which exhausts the buffering capacity of intracellular Ca^{2+} pools, particularly that of the mitochondria and endoplasmic reticulum, and triggers Ca^{2+}-mediated Ca^{2+}-release from intracellular stores (8). Damage to the mitochondria results in the enhanced production of free radicals, and in the translocation of "death factors" involved in apoptotic cell death into the cytosol. Accordingly, elevated $[Ca^{2+}]_i$ predisposes cells to self-degeneration (9).

Early diagnosis of AD would be very important, however it has proved to be a difficult task. Abnor-

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malities attributed to AD have been reported in many cell types such as erythrocytes (10), lymphocytes (11) and platelets (12). βAP also forms deposits in the skin of AD patients (13) presumably causing abnormalities in fibroblast biochemistry. A large number of studies have showed various alterations in fibroblasts, eg. disturbances of Ca^{2+}-uptake (14), glucose metabolism (15) and other biochemical changes (16,17). Peterson et al. (18) have found alterations in biochemical processes in cultured skin fibroblasts of Alzheimer donors.

In that fibroblasts have, for long time, served as models of neurobiological disorders (19) including AD (20), cutaneous biopsy has proved to provide great support in the diagnosis of neurodegenerative diseases (21,22). Our aim was to study whether readily available tissues such as fibroblasts can serve as simple supportive systems to the diagnosis of AD.

This communication summarizes techniques which have been used to study the biochemistry and physiology of skin fibroblasts isolated from patients with certain neurometabolic disorders including AD. Because AD has proved to be a systemic disorder, with the most prominent pathology in the cognitive functions of the brain, biochemical changes of fibroblasts are presumed to reflect changes that occur in the central nervous system of neurologically impaired patients (23).

EXPERIMENTAL PROCEDURE

Patients. Forearm excision biopsies were obtained after informed consent from late-onset sporadic AD patients (aged 73 ± 10.11, n_{AD} = 42) and age-matched controls (aged 71 ± 8.8, n_C = 16). At the time of skin biopsy, all participants were outpatients. The diagnosis of AD was made according to DSM-IV criteria (24). All AD donors met the DSM-IV definition of dementia of the Alzheimer's type, which requires evidence of cognitive deficits (criterion A) and a process of decline from previous levels (criterion C); none of them had history of familial AD. Control individuals were tested negative for any forms of dementia.

Fibroblast Culture Methods. All AD and control samples were cultured in the same conditions. For outgrowth of fibroblasts, small pieces of skin material were placed in cell culture flasks (25 cm^2) and grown for 3–5 weeks in Dulbecco's modified Eagle's medium (DMEM) containing 5% of heat inactivated fetal calf serum (FCS) as well as penicillin (100 U/ml), streptomycin (100 μg/ml) and 2 mM glutamate in a humidified atmosphere of 95% air and 5% of CO_2. Cells were detached for serial passaging using 0.01% of trypsin and 0.02% of EDTA. Stocks of each cell line (passage number: 5) were frozen to −80°C and stored at −130°C until use. After thawing, cells were used to seed on glass coverslips in a 24-multiwell plate in the DMEM medium containing 5% of FCS. Cultures maintained in this way were generally confluent by day 7 and were used for measurements of $[Ca^{2+}]_i$. All fibroblasts had a characteristic spindle-shaped appearance and were attached firmly to the coverslips. Viability of cells was estimated by the method of intravital staining with Trypan Blue after the incubation of cell suspension in the presence of the preparation (0.2% Trypan Blue in Hank's balanced saline solution, HBSS). After 5 minutes of incubation in the solution, 1.5% of cells absorbed the dye. In line with this finding we concluded that 98.5% of fibroblasts present on the coverslips were viable.

Measurement of Intracellular Free Calcium Levels. $[Ca^{2+}]_i$ were quantified by fluorescence ratio imaging of the calcium indicator dye Fura-2AM. Cells were incubated for 30 mins in the presence of 1 μM of the acetoxymethylester form of Fura-2AM. Cultures loaded with the dye were then washed twice with PBS solution and were used immediately for imaging. To check that loading of the cells with the dye and intracellular cleavage of the ester occurred, we sampled the fluorescence excitation ratios before loading the cultures with the dye. Ratios of unlabelled cells observed at 495 nm were 10.67 ± 3.26, around 3-times lower when compared to the ratio of 35.43 ± 8.03 after incubation with the dye; this is characteristic of Fura-2AM fluorescence. Images were obtained using a Hitachi F-2000 spectrofluorimeter. The ratio of the fluorescence emission of the cultures using two different excitation wavelengths of 340 nm and 380 nm, at emission wavelength of 495 nm was applied to determine $[Ca^{2+}]_i$. Total protein contents of the coverslips were measured according to Lowry (25).

Synthesis, Purification and Use of β-Amyloid (β[1–42]) Peptide in Fibroblast Cultures. The amyloid peptides were prepared by solid phase methodology (26) using Boc-chemistry with an ABI 430A automated peptide synthesizer on paramethylbenzhydrylamine(MBHA)-resin, using standard protocoll. Final deprotection as well as the cleavage of the peptides from the resin were performed with anhydrous hydrogen fluoride. After the removal of the hydrogen fluoride, the free peptides were precipitated with diethyl ether, filtered, washed with diethyl ether and extracted with 95% of TFA, diluted with water, and purified by using a preparative HPLC system (Shimadzu LC-8A, equipped with Bakbond WP C4 column, 300 × 47 mm, 300 Å pore size, 15–20 μm particle size). Peptides were eluted with a solvent system consisting of (1) 0.1% of aqueous trifluoroacetic acid and (2) 0.1% of trifluoroacetic acid in 80% of aqueous acetonitrile in a linear gradient mode for 30 minutes at 80ml/min flow. Pure fractions were pooled and lyophilized. Data of electrospray mass spectrometry (ES-MS, FinniganMat TSQ 7000 mass spectrometer) were in accordance with the calculated average molecular masses.

Peptides were dissolved in phosphate-buffered saline (PBS) and were incubated for an hour to "age" before direct use in cell culture. In the experiments where βAP was used, fibroblasts were incubated in 10^{-7}M of the peptide for 16 hours.

RESULTS AND DISCUSSION

Seven days after seeding, cultured fibroblasts from both Alzheimer and control donors were 80% confluent. Cells were competent to be labeled with Fura-2AM at 37°C by passive diffusion during the experiments. The ratio of fluorescence of the control cells at 340 and 380 nm representing $[Ca^{2+}]_i$ revealed 2.48 ± 0.162, whereas Alzheimer fibroblasts exhibited a ratio of 2.052 ± 0.207 (Table I). These findings indicate that cultured cells from donors of Alzheimer's disease demonstrate

Table I. 340/380 Fluorescence Excitation Ratios

Cultures	340/380 Fluorescence excitation ratio	Number of coverslips
Control	2.480 ± 0.162	16
Alzheimer	2.052 ± 0.207	42
Control + βAP	2.666 ± 0.08	16
Alzheimer + βAP	2.055 ± 0.125	42

Comparative fluorimetric studies on human control and Alzheimer fibroblasts on the 340/380 fluorescence excitation ratios using the Ca^{2+}-indicator dye Fura-2AM. The cells were cultured with or without β(1–42)amyloid peptide (βAP) for 16 hours at 37°C. The fluorescence excitation ratios were calculated from fluorescence intensities observed at 495 nm using excitation wavelengths of 340 and 380 nm.

significant decreases in free cell calcium when compared to that of age-matched controls. These data clearly indicate that detectable biochemical alterations are present in Alzheimer fibroblasts. A disrupted intracellular free calcium level of either fibroblasts or neurons, however, can be attributable to many metabolic disorders. To find specificity of these changes for AD, we investigated in another set of experiments whether abnormalities in response to long-term βAP exposition are also present in fibroblasts.

Measurements of $[Ca^{2+}]_i$ with Fura-2AM in control fibroblasts incubated for 16 hours in 10^{-7}M of fresh βAP[1–42] revealed an increase in the 340/380 fluorescence excitation ratio (2.66 ± 0.08), the mark of an increase in free calcium level (Table I). In contrast, Alzheimer cultures maintained in the same conditions showed little or no change in the free cell calcium (fluorescence ratio: 2.055 ± 0.125).

In order to rule out any possible artifacts caused by gross alterations in the cell number/cell mass actually present on the coverslips, the total protein content of the coverslips was also determined. Coverslips with control fibroblasts were found to contain 146.9 ± 8.4 μg protein/coverslip, whereas Alzheimer cells contained 139.1 ± 5.1 μg protein/coverslip. This difference is not significant in statistical terms, suggesting that the total protein content of Alzheimer and control fibroblasts, reflecting cell number, did not differ considerably.

Our data, summarized in Table I, indicate that chronic exposure of cells to βAP causes a rise in free cell calcium only in control fibroblast cultures. βAP disrupts calcium-regulating processes in control cells resulting in elevations of free cell calcium. Alzheimer fibroblasts do not show this change.

Despite the major pathological findings in the central nervous system of Alzheimer patients, the present study, as well as previous ones (10–19), indicate that metabolic deficits occur in non-neuronal tissues. Furthermore, fibroblasts express metabolic alterations that reflect dysfunction in other organs, such as the brain. The pathomechanism behind these findings remains unknown. In light of evidence, however, that βAP deposited in skin of patients with AD (13), we hypothesize that Alzheimer fibroblasts could be rendered resistant to βAP because of the chronic, long-term expositions, lasting years, of cutaneous cells to the peptide. These findings, however may also be attributable to agents other than βAP. This is there that our ongoing study aims to find specificity for alterations of $[Ca^{2+}]_i$, in AD.

The most common form of AD is of the late-onset sporadic type (27). Fibroblasts of our sporadic AD patients were distinguished from normal ones in our experiments. The specificity of our test for AD has not yet been proved, e.g., it can also be positive for Down-syndrome patients. However, our examination would appear useful for establishing new methods for the early diagnosis of late-onset AD cases.

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β-Amyloid-induced increase in the resting intracellular calcium concentration gives support to tell Alzheimer lymphocytes from control ones

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ABSTRACT: Senile plaques containing β-amyloid peptide (βAP) comprise the major neuropathological lesions in Alzheimer's disease (AD). In line with ongoing studies investigating alterations of various biochemical processes of cells of peripheral tissues, the authors demonstrate differences in resting intracellular free calcium levels of lymphocytes harvested from sporadic Alzheimer patients and from age-matched controls. Resting intracellular calcium concentration was measured in Fura-2AM-loaded human lymphocytes by dual wavelength spectrofluorimetry. Resting calcium level appeared to be higher in Alzheimer cells when compared to control lymphocytes. After incubating cells in 10^{-7} M of β-amyloid, the resting calcium concentration of the control cells elevated, while that of Alzheimer lymphocytes did not differ considerably. © 2002 Elsevier Science Inc. All rights reserved.

KEY WORDS: Alzheimer's disease, β-Amyloid, Lymphocyte, Fluorescence, Resting calcium.

INTRODUCTION

Alzheimer's disease (AD) is characterized histopathologically by the degeneration of specific populations of neurons and the accumulation of amyloid plaques within affected brain regions. The major component of the deposits is the β-amyloid peptide (βAP), a proteolytic fragment derived from the amyloid precursor protein (APP). Destabilization of neuronal Ca^{2+} homeostasis by βAP is considered central to the pathogenesis of AD [11].

Abnormalities attributed to AD have been reported not only in the brain, but also in many peripheral tissues, such as erythrocytes [15], platelets [17], lymphocytes [7], and other inflammatory objects [13,16,21] suggesting that this might be a systemic disorder with the most prominent pathology in the cognitive functions of the central nervous system [8]. Moreover, βAP, which arises from alternative processing of the ubiquitously expressed APP [18], is showed to form depositions in the skin of patients, with this kind of dementia [19], presumably causing abnormalities in fibroblast biochemistry. In our previous study [14], we have showed disruptions in intracellular ionic balance, particularly alterations in the calcium-homeostasis of fibroblasts, which mirror changes, thought to occur in the central nervous system of neurologically impaired patients [3]. The authors have found that cells from Alzheimer patients exhibited lower resting intracellular free calcium levels ($[Ca^{2+}]_i$) as compared to the control cultures. Our previous study [14] has also pointed out that exposure of fibroblasts to βAP resulted in increased $[Ca^{2+}]_i$ of the control cells, but not of AD fibroblasts. We have concluded that our test could prove useful in supporting the diagnosis of (sporadic) AD in patients suspected of suffering from the disease.

However, because of difficulties certain to be encountered with culturing fibroblasts harvested from aged individuals, the authors have investigated in this study whether readily available tissues such as lymphocytes of AD patients can serve as simple supportive systems for the diagnosis of AD.

MATERIALS AND METHODS

Patients

Blood samples were obtained after informed consent from late-onset sporadic AD patients (aged 71 ± 8.5 years [mean ± SD], $n_{AD} = 18$) and age-matched controls (aged 66.8 ± 10.2, $n_C = 23$). All participants were outpatients. The diagnosis of AD was made according to DSM-IV criteria [1]. All AD donors met the DSM-IV definition of dementia of the Alzheimer's type, which requires evidence of cognitive deficits (Criterion A) and a process of decline from previous levels (Criterion C); none of them had history of familial AD. Control individuals were tested negative for any forms of dementia.

None of the participants in our study received any medication known to interfere with calcium metabolism, such as antihypertensive drugs, calcium-antagonists, or antidepressants. None of the patients was on acetylcholinesterase inhibitor before and during the measurements. No probands suffered from any hematologic or inflammatory disorders.

Separation of Lymphocytes

Fresh blood samples were gently layered on Ficoll solution (9.56 g Ficoll, 20 ml pure iodamide, 130 ml dH_2O) and were centrifuged at 1800 rpm for 20 min. Lymphocyte-containing bands

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were rinsed with phosphate-buffered saline (PBS) three times. Cell concentration was estimated using Bürker's chamber. Freshly prepared lymphocytes in PBS were used for measurements of $[Ca^{2+}]_i$. Viability of cells was assessed by the method of intravital staining with Trypan Blue after the incubation of cell suspension in the presence of the preparation (0.2% Trypan Blue in Hank's balanced saline solution, HBSS).

Measurement of the Resting Intracellular Free Calcium Levels

$[Ca^{2+}]_i$'s were quantified by fluorescence ratio imaging of the calcium indicator dye Fura-2AM. Cells were incubated for 30 min in dark in the presence of 1 μM of the acetoxymethylester form of Fura-2AM. Lymphocytes loaded with the dye were then washed twice with PBS solution and were used immediately for measuring $[Ca^{2+}]_i$. Images were obtained using a Hitachi F-2000 spectrofluorimeter. The ratio of the fluorescence emission of the cells using two different excitation wavelengths of 340 and 380 nm, at emission wavelength of 495 nm was applied to determine $[Ca^{2+}]_i$. Absolute values of $[Ca^{2+}]_i$ were not calculated, in that in line with many other papers examining calcium levels [3,11,14] the authors found the rate of the fluorescence ratios to be more indicative of the change of the calcium level than the absolute figures. Total protein contents of the samples were assessed according to Lowry et al. [10].

Synthesis and Purification of β-Amyloid (β[1–42]) Peptide

The amyloid peptides were prepared by solid phase methodology [20] using Boc-chemistry with an ABI 430A automated peptide synthesizer on *para*-methylbenzhydrylamine (MBHA)-resin, using standard protocol. The completeness of acylation was monitored at each stage by the standard ninhydrin test [9]. Final deprotection as well as the cleavage of the peptides from the resin were performed with anhydrous hydrogen fluoride. After the removal of the hydrogen fluoride, the free peptides were precipitated with diethyl ether, filtered, washed with diethyl ether, and extracted with 95% of TFA, diluted with water, and purified by using a preparative HPLC system (Shimadzu LC-8A, equipped with Bakbond WP C4 column, 300 mm × 47 mm, 300 Å pore size, 15–20 μm particle size). Peptides were eluted with a solvent system consisting of (1) 0.1% of aqueous trifluoroacetic acid and (2) 0.1% of trifluoroacetic acid in 80% of aqueous acetonitrile in a linear gradient mode for 30 min at 80 ml/min flow. Pure fractions were pooled and lyophilized. Data of electrospray mass spectrometry (ES-MS, FinniganMat TSQ 7000 mass spectrometer) were in accordance with the calculated average molecular masses.

Throughout the study, only the βAP, synthesized by the authors, was applied on cultures. Freshly prepared aqueous βAP solutions were aged for 1 h before use. Cells were incubated for 2 h in 10^{-7} M final concentration of βAP. Fura2-AM was given to lymphocytes for 30 min in the last half-hour of exposition of cells to the amyloid.

Statistical Analyses

Data are given in mean ± SD. Student's *t*-probe was used for statistical calculations. All differences demonstrated herewith are considered significant, with $p < 0.05$.

RESULTS AND DISCUSSION

Lymphocytes were successfully harvested from both Alzheimer and control donors. Cells were competent to be labeled with Fura-2AM at 37°C by passive diffusion during the experiments. To check that loading of the cells with the dye and intracellular cleavage of the ester occurred, we measured the fluorescence excitation ratios before loading the cells with the dye. Ratios of unlabelled cells observed at 495 nm were 7.68 ± 2.2 (mean ± SD), around 10 times lower when compared to the ratio of 79.89 ± 10.11 after incubation with the dye; this is characteristic of Fura-2AM fluorescence. Values of the 340/380 fluorescence excitation ratio at 495 nm emission wavelength, which represents the $[Ca^{2+}]_i$, were computed after each fluorescence measurement. The ratio of fluorescence of the control cells at 340 and 380 nm was 1.001 ± 0.117, whereas Alzheimer lymphocytes exhibited a ratio of 1.131 ± 0.100 (Table 1). These data indicate that AD cells demonstrate increases in the resting free cell calcium when compared to cells of age-matched controls. These results are in line with the findings of Eckert et al. [4–6] and Bondy et al. [2].

TABLE 1

FLUORIMETRIC DATA OF LYMPHOCYTES FROM ALZHEIMER PATIENTS AND CONTROL INDIVIDUALS

Samples	340/380 Fluorescence excitation ratio (mean ± SD)	Number of cuvettes
Control	1.001 ± 0.117	23
Alzheimer	1.131 ± 0.100	18
Control + βAP	1.507 ± 0.368	23
Alzheimer + βAP	1.130 ± 0.075	18

Comparative fluorimetric measurements on lymphocytes of human Alzheimer and control donors on the 340/380 fluorescence excitation ratios using the Ca^{2+}-indicator dye Fura-2AM. Cells were maintained with or without β[1–42]AP (βAP) for 2 h at 37°C. The fluorescence excitation ratios were calculated from fluorescence intensities observed at 495 nm using dual wavelength spectrofluorimetry (excitation wavelengths: 340 and 380 nm).

Measurements of $[Ca^{2+}]_i$ with the calcium indicator dye Fura-2AM in control lymphocytes maintained in the presence of 10^{-7} M βAP[1–42] revealed an increase in the 340/380 fluorescence excitation ratio (1.507 ± 0.368), the mark of an increase in free calcium level during the 2-h exposure period. By directly monitoring $[Ca^{2+}]_i$ we found that Alzheimer cells, maintained in the same conditions, showed no significant change in the resting free cell calcium (fluorescence ratio: 1.130 ± 0.075).

Intravital staining was used to monitor the viability of the cells after fluorescence imaging. After 5 min of incubation with 0.2% of Trypan Blue, 1.9% of cells absorbed the dye, suggesting that 98.1% of lymphocytes present in the cuvettes were viable during the procedure.

In order to rule out any possible artifacts caused by gross alterations in the cell number/cell mass actually present in the cuvettes, the total protein content of the cuvettes was also determined. Cuvettes with control lymphocytes were found to contain 252.1±11.6 μg protein/cuvette, whereas Alzheimer cells contained 239.9 ± 14.0 μg protein/cuvette. This difference is not significant in statistical terms, so we concluded that the total protein content of Alzheimer and control lymphocytes did not differ considerably.

Lymphocytes of our sporadic AD patients were distinguished from normal ones in our experiments: AD cells exhibit higher $[Ca^{2+}]_i$ as compared to that of the controls. Exposure of control lymphocytes to βAP (10^{-7} M) can cause a rise in the resting free cell calcium; Alzheimer cells, however, appeared to be resistant to βAP (no significant alteration in $[Ca^{2+}]_i$). These data indicate that detectable biochemical alterations are present in fibroblasts [13] and lymphocytes of AD donors.

According to the previous experiments of the authors as well as Muller et al. [12], βAP can cause sustained alterations in cell membrane fluidity. Aggregated βAPs interact with membrane structures (both protein–peptide and lipid–peptide interactions), which

can cause permanent change in the structure of the cell membrane and in $[Ca^{2+}]_i$. These alterations might be the underlying mechanisms behind the resistance of lymphocytes to βAP with respect to $[Ca^{2+}]_i$.

Despite the major pathological findings in the central nervous system of Alzheimer patients, the present study, as well as previous ones [7,8,15,17,21] indicate that metabolic deficits occur in non-neural tissues, too. It is still unknown whether biochemical alterations seen with some peripheral tissues reflect the very dysfunctions in the brain, or these changes are specifically present only in the cell-types studied. The next step in our ongoing project is to prove that the findings demonstrated herewith are also characteristic of neurons. Because a disrupted resting intracellular free calcium level of either fibroblasts, lymphocytes or neurons can be attributable to many factors other than βAP, the authors also aim to find specificity for alterations of $[Ca^{2+}]_i$ in AD.

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Long-term exposition of cells to β-amyloid results in decreased intracellular calcium concentration

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Abstract

The ubiquitously present β-amyloid peptide plays an important role in the pathogenesis of Alzheimer's disease. Its neurotoxicity has been blamed on its mal-activity to increase calcium-levels. In the present study, we demonstrate that treatment of fibroblasts with β-amyloid has, in deed, resulted in a transient rise in the calcium-concentration. Chronic exposition of cultures to the peptide, however, caused a fall in the calcium-level. Apparently, β-amyloid has biphasic effects: acutely, it increases the calcium-concentration of cells; in contrast, on the long-run, β-amyloid peptide acts as a calcium-antagonist. Therefore, the idea that β-amyloid peptide leads to neural degeneration solely by increasing cells' calcium concentration must be replaced with a more complex view of its dual function in intracellular ionic homeostasis.

Keywords: Alzheimer's disease; β-Amyloid; Fibroblast; Calcium; Fluorimetry; Calcium-antagonist

1. Introduction

Senile plaques and neurofibrillary tangles comprise the major neuropathological lesions of Alzheimer's disease (AD). The plaques contain extracellular deposits of β-amyloid protein (βAP), which form abundant amyloid fibrils (7–10 nm) intermixed with amorphous aggregates of the peptide. Deposits are composed of βAP, the 42 amino-acid residue form of its precursor peptide. βAP is considered to play a key role in the pathophysiolgy of AD. Accumulation of βAP initiates inflammatory (Benveniste et al., 2001; Rogers and Lue, 2001; LaDu et al., 2001), oxidative (Bisaglia et al., 2002) and neurotoxic cascades. Progressive neuritic injury results in disruption of neural metabolic and ionic homeostasis. Destabilization of Ca^{2+} homeostasis in neurons plays a central role in neurodegeneration (Mattson et al., 1993).

The ubiquitously present neurotoxic βAP and its shorter fragments are deposited not only in certain regions of the brain, but also in several peripheral tissues. Apart from the central nervous system, abnormalities attributed to AD have been reported in many cell types such as erythrocytes (Perry et al., 1982), endothelial cells (Pákáski et al., 2002), lymphocytes (Eckert et al., 1998), and platelets (Sevush et al., 1998). Moreover, βAP, which arises from alternative processing of the ubiquitously expressed APP, is showed to form depositions in skin of patients with this kind of dementia (Soininen et al., 1992). This may imply that βAP causes abnormalities in intracellular biochemical pathways of peripheral cells. A large number of studies have showed various alterations in fibroblasts, e.g. disturbances of Ca^{2+}-uptake (Peterson et al., 1985), glucose metabolism (Sims et al., 1985) and other biochemical changes Paoletti and Tombaccini, 1998; Li and Kaminskas, 1985). Taken together, these observations may suggest that AD might be a systemic disorder with the most prominent pathology in the cognitive functions of the central nervous system.

In our previous studies, we have showed disruptions in intracellular ionic balance, particularly alterations in the basal intracellular calcium concentration $[Ca^{2+}]_i$ of cells of various peripheral tissues associated with AD, which mirror changes thought to occur in the central nervous system of neurologically impaired patients (Connolly, 1998). Also, we have previously pointed out that $[Ca^{2+}]_i$ of fibroblasts derived from AD patients is decreased when compared to that

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seen with age-matched controls Palotás et al., 2001). Resting calcium levels of Alzheimer lymphocytes, however, showed to be significantly higher than that of the controls (Palotás et al., 2002). Both Alzheimer lymphocytes and fibroblasts have proved to be resistant to βAP in our experiments.

In the present study, we demonstrate that treatment of fibroblasts with β-amyloid has, in deed, resulted in a transient rise in the calcium-concentration. Chronic exposition of cultures to the peptide, however, caused a fall in the calcium-level. Apparently, β-amyloid has a time-dependent dual effect: acutely, it increases the calcium-concentration of cells; in contrast, on the long-run, β-amyloid peptide acts as a calcium-antagonist. Therefore, the idea that β-amyloid peptide leads to neural degeneration solely by increasing cells' calcium concentration must be replaced with a more complex view of its dual function in intracellular ionic homeostasis.

2. Experimental procedures

2.1. Patients

Forearm excision biopsy was obtained after informed consent from a participant who was tested negative for any mental disorders or any forms of dementia, and was not institutionalized. The donor received no medication known to interfere with calcium metabolism, including calcium-antagonists, antihypertensive drugs, and antidepressants. The vlounteer had a negative family history of psychiatric diseases.

2.2. Culturing

2.2.1. Fibroblast culture methods

For outgrowth of fibroblasts, small pieces of skin material were placed in cell culture flasks (25 cm^2) and grown for 3–5 weeks in Dulbecco's modified Eagle's medium (DMEM) containing 5% of heat inactivated fetal calf serum (FCS) as well as penicillin (100 U/ml), streptomycin (100 μg/ml) and 2 mM glutamate in a humidified atmosphere of 95% air and 5% of CO_2. Cells were detached for serial passaging using 0.01% of trypsin and 0.02% of EDTA. After trypsinization, cells were used to seed on glass coverslips in a 24-multiwell plate in DMEM containing 5% of FCS. Cultures maintained in this way were generally confluent by day 9. All fibroblasts had a characteristic spindle-shaped appearance and were attached firmly to the coverslips. The seeding-and-passaging cycle was repeated throughout the study.

2.2.2. Viability

Viability of cells was estimated by the method of intravital staining with Trypan Blue after the incubation of cell suspension in the presence of the preparation (0.2% Trypan Blue in Hank's balanced saline solution, HBSS). After 5 min, 1.5% of cells absorbed the dye. In line with this finding, we concluded that 98.5% of cells were viable.

2.2.3. Treatment

Cells were cultured in the presence of 10^{-7} M of βAP. The authors used this molarity during the experiments in that the concentration of βAP in the serum of Alzheimer patients is 10^{-7} M. Control cultures were free of any treatment.

2.3. Measurement of intracellular free calcium levels

$[Ca^{2+}]_i$ were quantified by fluorescence ratio imaging of the calcium indicator dye Fura-2AM. Cells were incubated for 30 min in the presence of 1 μM of the acetoxymethylester form of Fura-2AM. Cultures loaded with the dye were then washed twice with PBS solution and were used immediately for measuring the fluorescence intensities. Images were obtained using a Hitachi F-2000 spectrofluorimeter. The ratio of the fluorescence emission of the cultures using two different excitation wavelengths of 340 and 380 nm, at emission wavelength of 495 nm was applied to determine $[Ca^{2+}]_i$. In line with many other papers examining calcium levels (Connolly, 1998; Palotás et al., 2001, 2002; Laskay et al., 1997), the authors found the rate of the fluorescence ratios excellent for monitoring alteration of $[Ca^{2+}]_i$, without calculating actual $[Ca^{2+}]_i$.

To monitor that labeling of the cells with the dye and intracellular cleavage of the ester occurred, we sampled the fluorescence excitation ratios before loading the cultures with the dye. Ratios of unlabeled cells observed at 495 nm were 10.67 ± 3.26, around three-times lower when compared to the ratio of 35.43 ± 8.03 after incubation with the dye; this is characteristic of Fura-2AM fluorescence.

No significant alteration was detectable among labeled cultures in Fura-2AM fluorescence using the calcium-insensitive excitation wavelength of 367 nm, indicating that the observed response reflects a real change in the calcium-level of the cells.

Because gross alterations in the cell number per cell mass actually present in the cuvettes may interfere with the fluorescence intensities, the total protein content of the cuvettes was determined according to Lowry et al. (1951). Cuvettes were found to contain 241.6 ± 12.9 μg protein per cuvette, with no cuvette significantly deviating from the mean; this suggests that the total protein content of the cultures examined did not differ considerably.

2.4. Synthesis and purification of β-amyloid(1–42) peptide (βAP)

Solid phase protein synthesis was used for the preparaion of βAP (Stewart and Young, 1984). Peptides were purified by a Shimadzu LC-8A preparative HPLC system. Pure βAP's were dissolved in phosphate-buffered saline (PBS) and were incubated for an hour to "age" before direct use in the cultures.

Table 1
The 340/380 fluorescence excitation ratios of fibroblast cultures

	Resting level	8 h	~Half-week	~16 weeks	~27 weeks	~37 weeks
βAP	2.479 ± 0.221	2.691 ± 0.121	2.798 ± 0.101	2.802 ± 0.157	2.164 ± 0.199	2.165 ± 0.176
Control	2.479 ± 0.221	2.483 ± 0.107	2.475 ± 0.200	2.489 ± 0.113	2.469 ± 0.132	2.489 ± 0.206

Comparative fluorimetric studies on human fibroblasts on the 340/380 fluorescence excitation ratios using the Ca^{2+}-indicator dye Fura-2AM. The cells were cultured with β-amyloid(1–42) peptide (βAP) for 23 weeks at 37 °C. The fluorescence excitation ratios were calculated from fluorescence intensities observed at 495 nm using excitation wavelengths of 340 and 380 nm.

2.5. Data analysis

Values are given as arithmetic mean ± standard deviation. All differences stated in the text are statistically significant (student's *t*-tests, $P < 0.05$).

3. Results and discussion

Short-term (8 h) exposure of cultures to βAP resulted in elevation of the fluorescence ratio (FL: 2.691±0.121), which is an indicative of the increase in the $[Ca^{2+}]_i$ (Table 1). This finding is in line with previous reports, demonstrating that a sustained rise in the basal calcium level is one of the underlying pathomechanisms of neural degeneration (Palotás et al., 2001, 2002; Laskay et al., 1997).

In our long-term study, the βAP-induced increase in the $[Ca^{2+}]_i$ peaked on the 4th day of exposure to the peptide (FL: 2.798 ± 0.101), and turned into a plateau for around 16 weeks (Fig. 1). After the approximately 4 months' time the calcium-level of the cells gradually started to decrease. Within 11 weeks, the $[Ca^{2+}]_i$ became significantly lower in the cultures studied (FL: 2.164 ± 0.199) when compared to that of the resting levels seen with cultures before or without exposure to βAP (FL: 2.479 ± 0.221) (Table 1). The decreased calcium-level appeared to be constant for 10 weeks.

During the experiments, the treated and control cell populations were cultured in media of identical composition except for the presence or absence of the β-amyloid peptide. Therefore, it is reasonable to conclude that the observed difference in the 340/380 fluorescence excitation ratio of Fura-2AM-loaded cells is only associated with the presence or absence of β-amyloid peptide, and hence can be regarded as an effect induced by the β-amyloid peptide itself.

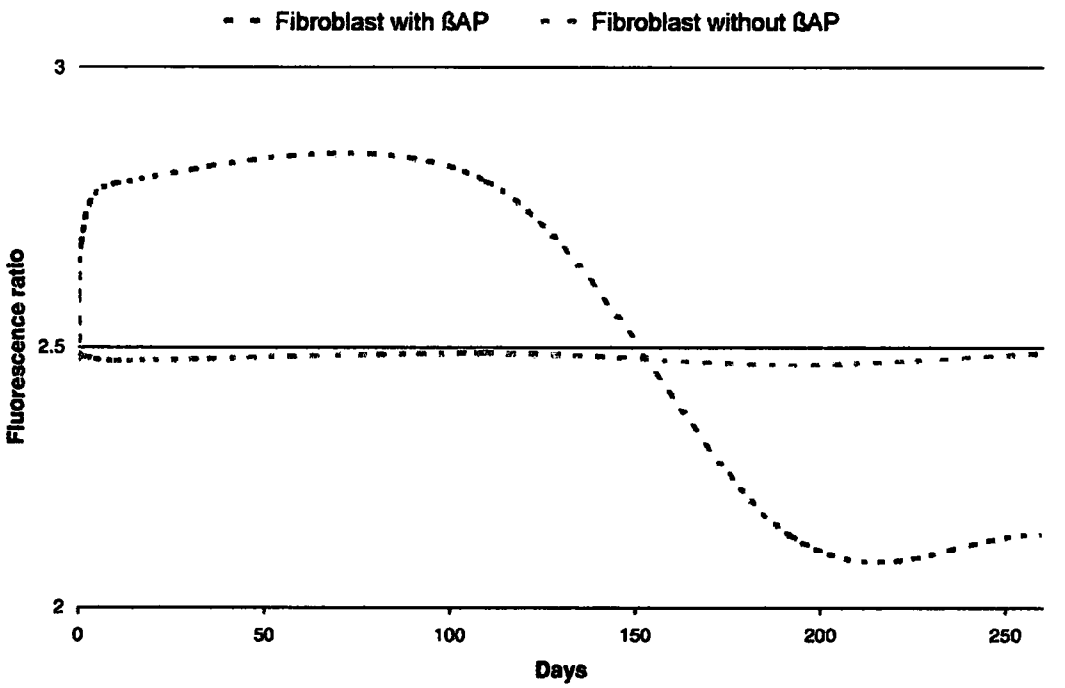

Fig. 1. Time-course of the calcium-level of human fibroblasts during chronic βAP treatment. Short-term exposition of fibroblast cultures to βAP results in an increased $[Ca^{2+}]_i$. The rise in the calcium-level peaks after an approximately half-a-week treatment with the peptide. The flow-chart demonstrates that the incessant elevation of $[Ca^{2+}]_i$ turns into a fall at around the 16th week of exposition to βAP, and yields a sustained decrease in the $[Ca^{2+}]_i$. Calcium-level of control cultures with no βAP treatment appeared to be unchanged during the experiment.

Cells in our study have responded to β-amyloid treatment in a time-dependant manner. Consistent with previous findings (Palotás et al., 2001, 2002; Laskay et al., 1997), $[Ca^{2+}]_i$ is increased as a result of βAP; this, however, seems to be only a short-term effect of the peptide. Piling evidence suggests that βAP interacts with cell membrane structures, including ion channels, and also causes changes in membrane fluidity (Muller et al., 1995). Exposure to βAP elicits sustained activation of Ca^{2+}-permeable receptor channels, which results in a pathological enhancement of inward Ca^{2+} currents and a subsequent increase in the $[Ca^{2+}]_i$ (Harkany et al., 2000; Mattson, 1997; Mogensen et al., 1998; Stix and Reiser, 1998). Elevation of $[Ca^{2+}]_i$ may further progress through the activation of second messengers (e.g. IP_3) that mobilize intracellular calcium ions resulting in Ca^{2+}-release from its intracellular stores (Cowburn et al., 1995; Kimura and Shubert, 1993 Ishikawa et al., 1998). We hypothesize that the acute rise in $[Ca^{2+}]_i$ is attributable to these interactions of βAP.

On the other hand, chronic exposition of cells to βAP apparently decreases $[Ca^{2+}]_i$. This observation is a real novum, and the pathomechanism behind this finding remains unknown. Falling of the calcium-level may indicate that βAP causes a total ionic imbalance. We speculate that $[Ca^{2+}]_i$ may reach pathological concentrations which exhausts the buffering capacity of intracellular Ca^{2+}pools, particularly that of the mitochondria and endoplasmic reticulum, and triggers Ca^{2+}-mediated Ca^{2+}-release from intracellular stores (Pascale and Etcheberrygaray, 1999). At later stages, damage to the mitochondria results in the enhanced production of free radicals, and in the translocation of "death factors" involved in apoptotic cell death into the cytosol. Due to this process, and also to their direct chronic interaction with βAP, calcium channels may become hyperactive, causing an incessant outward calcium flow. This can not be controlled by the cells' internal buffer systems on the long term, yielding a sustained decrease in the $[Ca^{2+}]_i$. Accordingly, chronic fall in $[Ca^{2+}]_i$, along with the accumulation of apoptotic factors, predisposes cells to self-degeneration (Pascale and Etcheberrygaray, 1999). As a result, derange of the intracellular calcium homeostasis ensues, and cells ultimately succumb to the chronic ionic imbalance. Taken together, we conclude that the neurotoxicity of βAP is due to its dual effect, but mainly to its long-term calcium-antagonistic activity.

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RISPERIDON ANTAGONIZÁLJA A β-AMYLOID PEPTID OKOZTA INTRACELLULÁRIS KALCIUM-SZINT VÁLTOZÁSOKAT FIBROBLASZTOKON

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ÖSSZEFOGLALÁS

Az Alzheimer-kóros betegek egy részénél megnyilvánuló viselkedési zavarok kezelésére antipszichotikumok alkalmazására van szükség. Kevés információval rendelkezünk azonban arra vonatkozólag, hogy a neuroleptikum-kezelés miként hat a demencia lefolyására. Mivel a kór kialakulásáért felelősnek tartott β-amyloid peptid sejtkárosító, ion-háztartást felborító hatása fibroblasztokon is kimutatható, arra kerestünk munkánk során választ, hogy a gyakran használt atípusos antipszichotikum, a risperidon, hogyan befolyásolja a könnyen tenyészthető szövetek, úgy mint a fibroblasztok kalcium-egyensúlyát. Fluorimetriás módszerrel tanulmányoztuk az Alzheimeres és kontroll fibroblasztok ionikus homeosztázisát, valamint ß-amyloid peptidre, illetve risperidonra adott válaszát. Az Alzheimer-kóros betegekből származó fibroblasztok intracelluláris nyugalmi szabad kalcium szintjei alacsonyabbak voltak a kontrollokhoz viszonyítva. Kontroll fibroblasztok emelkedett kalcium-szinttel válaszoltak a β-amyloid hatására, míg az Alzheimeres tenyészetek esetében ez a változás elmaradt. Risperidon hatására azonban a β-amyloid-peptid kalcium-szintet emelő hatása nem volt megfigyelhető. A β-amyloid peptid kórosan megemeli a fibroblasztok kalcium szintjét; risperidon *in vitro* hatékonyan antagonizálja a peptid ezen sejtkárosító hatását.

KULCSSZAVAK: Alzheimer-kór – β-amyloid peptid – fibroblaszt – fluoreszcencia – risperidon

RISPERIDONE ANTAGONIZES β-AMYLOID PEPTIDE-INDUCED INTRACELLULAR CALCIUM LEVEL CHANGES IN FIBROBLASTS

Antipsychotics are widely used in the management of behavioral and psychiatric symptoms of Alzheimer's disease; however, little information is available on the effect of these drugs on the progression of dementia. The neurotoxic β-amyloid peptide, the causative agent in Alzheimer's disease, leads to ionic imbalance in both neurons and fibroblasts. Therefore, the purpose of our study was to monitor the impact of the frequently prescribed antipsychotic, risperidone, on the calcium homeostasis of readily available tissues, such as fibroblasts. Calcium levels of primary fibroblast cultures harvested from Alzheimer patients and age-matched controls were measured utilizing fluorimetric studies after treatment with β-amyloid peptide and/or risperidone. Intracellular basal calcium concentration of Alzheimer fibroblasts was lower when compared to that of controls. β-amyloid peptide caused an increase in the calcium level of control cultures only, leaving the ionic homeostasis of Alzheimer cells intact. Co-administering β-amyloid peptide with risperidone did not change the calcium concentration considerably. β-amyloid peptide elevates calcium concentration of fibroblasts to toxic levels. Administration of risperidone efficiently attenuates β-amyloid-induced cytotoxicity *in vitro*.

KEYWORDS: Alzheimer's disease – β-amyloid peptide – fibroblast – fluorescence – risperidone

BEVEZETÉS

Az Alzheimer-kórban (AD) bekövetkező neuropathológiai elváltozásokért valószínűleg a β-amyloid peptid (βAP) felelős. Ez a neurotoxikus ágens többek között az idegsejtek ionikus homeosztázisának felborításával, azon belül is főleg az intracelluláris Ca^{2+}-szint emelésével okozhatja a neuronok degenerációját (1).

Ez a biokémiai elváltozás nemcsak az idegsejtekben, hanem más perifériás szövetben is kimutatható: AD-betegek lymphocytái és fibroblasztjai felborult kalcium-egyensúlyt mutatnak – feltehetőleg a krónikus βAP-expozíció következtében (2,3). Egyre több adat jelzi, hogy az AD egy szisztémás megbetegedés, mely dominánsan a központi idegrendszert érinti (4).

Az AD kísérő tüneteinek jelentős részét viselkedési zavarok alkotják. A betegek több mint felénél figyelhető meg pszichózis, delírium, agitáció, stb., melyek kezelésében a tradicionális antipszichotikus terápia mellett újabban a magas potenciálú atípusos szerekkel, pl. risperidonnal, kényegében mellékhatásoktól mentesen, kívülő eredmények érhetők el (5-8).

Retrospektív összehasonlító tanulmányok kimutatták, hogy szkizofréniában szenvedő betegek esetében az Alzheimer-kór előfordulási gyakorisága alacsony (7). Ennek feltételezhetően kóroki magyarázata is lehet, azonban egyre több jel utal arra, hogy ez a viselkedési zavarokban alkalmazott neuroleptikumoknak köszönhető (9-12). Az epidemiológiai megfigyelés viszont eddig még nem nyert közvetlen bizonyítékot.

Kísérletünk fő céljaként az AD és kontroll egyénekből származó fibroblasztokat vizsgáltuk. Arra kerestük a választ, hogy miként befolyásolja a βAP által felborított intracelluláris nyugalmi szabad kalcium koncentrációt ($[Ca^{2+}]_i$) egy magas potenciálú atípusos antipszichotikum, a risperidon. Munkánk során tehát azt tanulmányoztuk, hogy a fibroblasztokon található biokémiai elváltozásokat – melyek tükrözik a neuronokban korábban leírt degenerációs folyamatokat az ionháztartás szempontjából (1-3) – hogyan befolyásolja a risperidon kezelés.

ANYAGOK ÉS MÓDSZEREK

Donorok

Az etikai engedély megszerzését követően késői típusú sporadikus AD betegek (életkoruk: 73±10.11 év, esetszám: N_{AD}=14) és korban összevethető kontrollok (koruk: 71±8.8 év, N_K=8) alkarjából előzetes beleegyezés után bőrbiopsziát nyertünk. Az AD klinikai diagnózisa és a kontrollok bármilyen típusú demenciájának kizárása a BNO-10 és DSM-IV kritériumok szerint történt (13). Minden donor esetében a beavatkozást ambuláns módon végeztük, és egyikükön sem igazolódott semmiféle neurológiai-pszichiátriai társbetegség jelenléte. A rutin laboratóriumi tesztek eredményei a mindkét csoport esetében a normál tartományokon belül voltak.

Fibroblasztok tenyésztése

A biopsziával nyert szövetdarabkákat 3-5 hétre Dulbecco's modified Eagle's medium (DMEM)-oldatba helyeztük, mely 5% fetal calf serumot (FCS), penicillint (100 U/mL), streptomycint (100 µg/mL) és glutamint (2mM) is tartalmazott. A fibroblasztokat 5%-os CO_2 mellett növesztettük. A passzálás 0.01%-os trypsin + 0.02%-os EDTA-t tartalmazó oldattal történt. Az ötödik tripszinizálás után a tenyészeteket lefagyasztottuk és a további felhasználásig –130°C-on tároltuk. Kiolvasztás után a fibroblasztokat fedőlemezekre helyeztük, és 5%-os FCS-t tartalmazó DMEM-ben szuszpendáltuk fel.

A sejtek működőképességének mérése

Méréseink után a tenyészetek életképességét Trypan-kék intravitális festékkel ellenőriztük: 5 perces inkubálás után a fibroblasztok 1.5%-a vette fel a festéket, mint azt jelzi, hogy a fedőlemezeken található sejtek 98.5%-a volt életképes.

Protein-tartalom meghatározása

A tenyészetek esetlegesen eltérő sejtszámából adódó mérési hibák kiküszöbölése céljából összehasonlítottuk az egyes fedőlemezek fehérje mennyiségét. A fedőlemezeken található tenyészetek össz-fehérje tartalmát Lowry módszerével határoztuk meg (14). A kontroll tenyészetek 146.9±8.4 µg/fedőlemez, míg az Alzheimeresek 139.1±5.1 µg/fedőlemez protein tartalmúnak. Ez a különbség statisztikailag elhanyagolható, így arra a következtetésre jutottunk, hogy az egyes tenyészetek fehérjetartalma lényegesen nem tért el. Ez azt jelenti, hogy a fluoreszcenciák értékeit a sejtszámbeli különbségek nem befolyásolhatták. A mérési eredmények tehát hűen tükrözik a kezelések hatását.

A $[Ca^{2+}]_i$ mérése

Az $[Ca^{2+}]_i$-t fluoreszcens módszerrel határoztuk meg Münchi P-2000 spektrofluorimétereri. A sejtek $[Ca^{2+}]_i$-je arányos az 525 nm-en mért, 340 és 380 nm-eken gerjesztett fluoreszcenciák hányadosával. A tenyészeteket kalcium-szenzitív Fura-2AM oldattal (1µM) inkubáltuk fél órán át, majd 2x phosphate-buffered saline (PBS) öblítés után közvetlenül meghatároztuk a két gerjesztési értéknél a fluoreszcenciát.

Azokat a mérési sorozatoknál, amikor a βAP, illetve a risperidon hatását vizsgáltuk, először a peptiddel és/vagy farmakonnal inkubáltuk a sejteket 16-órán keresztül, majd az utolsó 30 percben adtuk a mintákhoz a kalcium-indikátor Fura-2AM festéket; ezt követően 2xPBS mosás után hasonlóképpen elvégeztük a méréseket. A tenyészetek βAP-koncentrációját az Alzheimeres betegek szöveteiben található βAP-szintnek megfelelően 10^{-7}M-nak választottuk. Risperidont a szer terápiás vérszintjével megegyező koncentrációban alkalmaztuk.

Az egyes betegekből származó mintákból többszöri méréseket végeztünk (n_{AD}=42, n_K=16).

A βAP szintézise és tisztítása

Fam-methylbenzhydrylamine(MBHA)-gyantán, szilárd fázisú peptidszintézissel történt a βAP előállítása. Boc-kémiát alkalmazva, standard protokoll szerint jártunk el (15). A szintézishez ABI

430A automata peptidszintetizátort használtunk. A deprotekciálás és a gyantáról való leválasztás hidrogén-fluoriddal történt. Ezt követően a nyers anyagot HPLC-vel tisztítottuk (Shimadzu LC-8A, Nucleosil WP C4 oszlopokkal: 300x47mm, 300 Å pórus átmérő, 15-20 µm particulum méret). A tiszta βAP frakciókat lyophilizáltuk, majd felhasználáskor PBS-ben oldottuk fel.

Gyógyszerek

A risperidont kereskedelmi úton szereztük be (Risperdal®; Janssen Cilag, Bis-Int-63, Ref. No.: 336341). A farmakont a mellékelt steril, intravénás felhasználásra szánt oldatában szolubilizáltuk, és közvetlenül alkalmaztuk a tenyészeteken 50 µg/l koncentrációban, mely megfelel a szer terápiás vérszintjének (16-17).

Statisztikai számítások

Méréseink eredményeit átlag±standard deviáció formátumban tüntettük fel. Statisztikai számításainkat (a szignifikancia megállapításakor) *t*-próbát alkalmaztunk. A változásokat $p<0.05$ esetén tekintettük szignifikánsnak.

EREDMÉNYEK, KÖVETKEZTETÉSEK

Alzheimer-kóros és idős kontroll egyének bőrbiopsziából származó fibroblasztokat sikeresen tudtunk fodlemezekre tenyészteni, és kísérleteinkben alkalmazni. Fura-2AM festékkel végzett méréseink során azt tapasztaltuk, hogy a kontroll tenyészetek (n_{KO}=16) 340/380nm-es fluoreszencia-hányadosa ($FI_{340/380}$), mely reprezentatívan tükrözi a $[Ca^{2+}]_i$-t, 2.48±0.162 volt. Ezzel szemben az AD sejtek (n_{AD}=42) esetén ez az érték szignifikánsan alacsonyabb ($FI_{340/380}$: 2.052±0.207). Eredményeink tehát arra utalnak, hogy a kontroll fibroblasztok $[Ca^{2+}]_i$-ja magasabb, mint az AD sejteké (1. táblázat). Hasonló eltéréseket csökkent nyugalmi kalcium szintet talált Peterson és mtsai AD fibroblasztokon (18-19). Mivel a βAP képes megváltoztatni a neuronok ionikus anyagcseréjét a kalcium szint emelésével (1), ezzel a peptiddel kezeltük sejtjeinket, hogy megvizsgáljuk érzékenységüket az amyloidra vonatkozóan.

Tizenhat órás, 10^{-7} M-os amyloid kezelés hatására a kontroll sejtekben mért $FI_{340/380}$ szignifikánsan megnőtt (értéke: 2.66±0.08), mely arra utal, hogy a βAP megemeli a fibroblasztok $[Ca^{2+}]_i$-t. Ez összhangban áll az irodalmi adatokkal, melyek a βAP $[Ca^{2+}]_i$-emelő hatását hangsúlyozzák neuronokon (1). Ezzel szemben azonban az Alzheimeres mintákban észlelt fluoreszcenciaarány ($FI_{340/380}$: 2.055±0.125), így a sejtek $[Ca^{2+}]_i$-ja, szignifikáns eltéréseket nem mutatott (1. táblázat).

A kísérleteinkben észlelt eltéréseket az irodalmi adatokkal összevetve kontrollált tendenciát fedezhetünk fel. Eddigi kutatások is azt tanúsítják, hogy az AD fibroblasztokban biokémiai eltérések találhatók, például a glükóz-metabolizmus és kalcium-felvétel zavarai (18, 20-21). Neuronokon végzett mérések további azt bizonyítják, hogy βAP hatására megnövekszik a kalcium átengedő képesség, és következményes ionáram indul meg a sejt belseje felé (22-23). A kissé megnőtt kalcium szint, mint központi másodlagos hírvivő, számos úton tovább emelheti a sejt kalcium koncentrációját (24-26), miellyel az intracelluláris puffer-rendszerek nem tudnak egyensúlyt tartani. Ennek hatására szabadgyökök halmozódnak fel, mely a sejt pusztulásához vezethet (18). Véleményünk szerint ez a folyamat mehet végbe Alzheimeres fibroblasztokban is.

Eredményeink értelmében a "nyugalmi" $[Ca^{2+}]_i$ értékek alapján az AD betegek fibroblasztjai megkülönböztethetők a kontroll sejtektől. Hosszú távú βAP kezelésre a nem demens egyénekből származó tenyészetek Ca^{2+}-szint emelkedéssel válaszoltak; az amyloid tehát a sejtek ionikus homeosztázisának felborításával befolyásolja az $[Ca^{2+}]_i$-t. Alzheimeres sejtek esetében ezt a változást azonban nem láttuk. Hogy az AD fibroblasztok csökkent βAP válasza a krónikus amyloid hatás következtében kialakuló deszenzitizáció miatt jön létre, vagy más oka van, jelenleg még nem ismert.

Risperidon kezelés során a szer szignifikáns eltérést nem okozott sem a kontroll ($FI_{340/380}$: 2.479±0.200), sem az AD tenyészetekben ($FI_{340/380}$: 2.054±0.172) a kezeletlen mintákhoz képest. A farmakon viselkedési zavarokban történő használata tehát biztonságos lehet a sejtek intracelluláris kalcium-anyagcseréje szempontjából. Az atípusos antipszichotikummal együtt alkalmazott βAP kezelés esetén jelentős eltérés az $[Ca^{2+}]_i$-ben szintén nem volt megfigyelhető a kontroll mintákhoz viszonyítva (1. táblázat). Ez azt jelenti, hogy a risperidon nemcsak biztonságos az $[Ca^{2+}]_i$-ra vonatkozóan, de a βAP ionháztartást felborító hatását is kivédi. Fibroblasztokon végzett kísérleteink alapján tehát a risperidon a citotoxikus βAP-vel szemben sejtvédő hatással is rendelkezik.

Irodalmi adatok eddig még nem számoltak be a risperidon és a Ca^{2+} közti kapcsolatról, valamint arról sem, hogyan befolyásolja ezen ion a βAP, illetve annak prekurzorának metabolizmusát. Ismert azonban, hogy dopamin-receptorok aktivációja tranziens $[Ca^{2+}]_i$-emelkedést okoz. Atípusos szerekről, úgy mint a risperidonról, leírták, hogy ezen receptorok blokkolásával a $[Ca^{2+}]_i$-szintet stabilizálják, valamint a D_1/D_2-receptorok dopamin és egyéb agonistái iránt de-

szenzitizációját váltják ki (27), feltehetőleg tartós Ca^{2+}-egyensúlyt hozva így létre. Dopamin-receptor antagonistákról továbbá kimutatták, hogy – éppúgy, mint az antioxidánsok – a βAP neurotoxikus hatását kivédik (28). Ezen adatok alapján feltételezzük, hogy a risperidon munkánk során észlelt sejtvédő tulajdonsága tehát a D_2-hatásból származhat.

A kór extenzív kutatása alapján, mely mind a központi idegrendszerre, mind a perifériára irányul, egyre több adat támasztja alá, hogy az AD szisztémás megbetegedés, mely demencia képében manifesztálódik (4). Ezen megfigyelések nyitották még a zöld sávot ahhoz, hogy az AD biokémiai elváltozásait a perifériás szövetek tanulmányozásával vizsgálhassuk (29-33). Az Alzheimer-kóros betegek esetében a központi idegrendszeren kívül észlelt biokémiai eltérések több tekintetben tükrözik az agyban észlelhető defektusokat (33-34); ezek komplett patomechanizmusa még a mai napig is ismeretlen. A fibroblasztokban észlelt elváltozásokat modellként használva (33-36) azonban feltételezhető, hogy a risperidon-kezelés során a periférián észlelt protektív folyamatok a központi idegrendszerben is megfigyelhetők. Amennyiben ez a megállapítás a későbbiekben bizonyítékot nyer, a risperidon – vagy hasonló molekulák – alkalmassá válhatnak nemcsak a viselkedési zavarok, de maga az Alzheimer-kór kezelésére is, illetve kiindulási vegyület lehet ilyen jellegű gyógyszerek kifejlesztésében.

I. táblázat. A nyugalmi és β-amyloid, valamint risperidon kezelés utáni kalcium-szintek fibroblasztokban

Tenyészetek	$FL_{340/380}$	Lemez-szám
Kontroll	2.48±0.162	16
AD	2.052±0.207*	42
Kontroll+βAP	2.666±0.08*	16
AD+βAP	2.055±0.125	42
Kontroll+RIS	2.479±0.200	16
AD+RIS	2.054±0.172	16
Kontroll+βAP+RIS	2.482±0.114	16
AD+βAP+RIS	2.053±0.099	16

Rövidítések:
AD: Alzheimer kóros betegektől származó fibroblasztok; βAP: β-amyloid peptid; $[Ca^{2+}]_i$: intracelluláris szabad kalcium szint; $FL_{340/380}$: 340/380nm-en gerjesztett, 525nm-en mért fluoreszcenciák hányadosa; RIS: risperidon; *: $p<0.05$ (ahol 'p': szignifikancia-szint).

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A szerzők ezúton szeretnék megköszönni a Szegedi Tudományegyetem Sebészeti Műtéttani Intézet munkatársainak a spektrofluoriméter használatának lehetőségét. Hálával tartozunk továbbá a Szegedi Tudományegyetem Bőrgyógyászati Klinikájának a fibroblaszt tenyészetekért.
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Effect of haloperidol and risperidone on amyloid precursor protein levels in vivo

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Abstract

The neurotoxic β-amyloid peptide of Alzheimer's disease is formed from the amyloid precursor protein (APP), which is a member of an evolutionarily highly conserved gene family with significant functional importance. Because behavioral and psychiatric symptoms treated with antipsychotics may influence the course of the disease, we have investigated traditional and atypical antipsychotic drugs, administered through the intraperitoneal route, for their effects on rat cortical APP. Western-immunoblotting was utilized for semi-quantitative evaluation of APP levels. Treatment with haloperidol resulted in an acute elevation of cortical APP both in therapeutic and toxic doses, however, it had no significant chronic impact on APP. Atypical antipsychotic risperidone did not change cortical APP concentration. These results indicate that both haloperidol and risperidone are considered to be relatively safe with respect to APP metabolism. Possible mechanisms, including involvement of calcium and APP itself as a receptor, are discussed.

Keywords: Amyloid precursor protein; Antipsychotic; Calcium; Haloperidol; Receptor; Risperidone

1. Introduction

The amyloid precursor protein (APP) is a large, ubiquitously expressed, membrane spanning glycoprotein which is endoproteolytically processed to a 4-kDa, 39–43 amino acid residue, called the β-amyloid peptide (βAP), that is the major component of senile plaques in Alzheimer's disease (AD) [44].

The major or normal route of APP processing is via the α-secretase pathway, which is incompatible with the formation of βAP. This non-amyloidogenic metabolism yields neuroprotective, soluble form of APP (sAPPα) [20]. The amyloidogenic pathway is an alternative of APP processing, involving cleavage of the precursor by β- and γ-secretases [23]; as a result, neurotoxic βAP is produced.

The strong structural conservation within the superfamily and ubiquitous expression within the body indicate an important function for APP and APP-like proteins [15]. They play a role in the regulation of neurite growth, intracellular calcium levels, cytokine release, promotion of neuronal survival, and cell adhesion in neuronal cells [31]. It has also been proposed that APP is a cell-surface receptor [27]. However, a specific soluble ligand that binds to surface-inserted APP with receptor-like kinetics has not been identified. Potential support for a receptor-like function of APP is the observation that the precursor can associate with the GTP-binding protein G_o via a motif in the APP cytoplasmic domain [36].

Just as APP is essential for the organism's survival, disturbance of its regulation appears to participate in this astounding human disease, AD, by yielding freely floating βAP. During the course of the disorder, AD patients may develop behavioral and psychiatric symptoms of dementia (BPSD) that require antipsychotic medication. Recent reports demonstrate that AD pathology is infrequent in patients with schizophrenia (SCH) [2,12], and it has been proposed that antipsychotic medications used to treat SCH

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might actually have a protective effect against developing AD neuropathology.

Traditional antipsychotic haloperidol (HAL) is dopamine-2 (D_2) and sigma-1 (σ_1) receptor antagonist with apoptotic activity [5,22,47]. HAL is involved in neurotoxicity, causing clinically troublesome adverse events such as extrapyramidal and cardiac side-effects [3]. HAL was also demonstrated to efficiently inhibit βAP formation from APP in vitro [25]. The mechanism by which it might exert such an effect is not known. On the other hand, atypical antipsychotic risperidone (RISP) is a high potential D_2, serotonine (5-HT_2) and noradrenergic (NA) antagonist with less adverse effects then conventional antipsychotics. RISP has no known effect on AD pathology, including APP metabolism.

In light of evidence that HAL—one of the most frequently prescribed antipsychotic medications used in the treatment of SCH [7]—interferes with APP processing, we evaluated HAL for its effects on APP metabolism in vivo; we have also examined RISP in this regard.

2. Materials and methods

2.1. Animals

Male, Sprague–Dawley rats weighing 200–250 g were maintained under standard laboratory conditions. With respect to haloperidol (Haloperidol®; Gedeon Richter Ltd., Hungary), groups of six rats were injected i.p. on a daily basis with therapeutic (0.05 mg/kg) or toxic (0.5 mg/kg) doses dissolved in saline [10,18]. In case of risperidone (Risperdal®; Janssen-Cilag, division of Johnson & Johnson Ltd., Hungary), therapeutic (0.1 mg/kg) or toxic (1.0 mg/kg) doses, dissolved in water, were administered [8,17]. Control rats received the vehicle alone. No other animals were housed in the room or allowed contact with the study animals.

Experiments were performed in accordance with a protocol approved by the university ethics committee on laboratory animals.

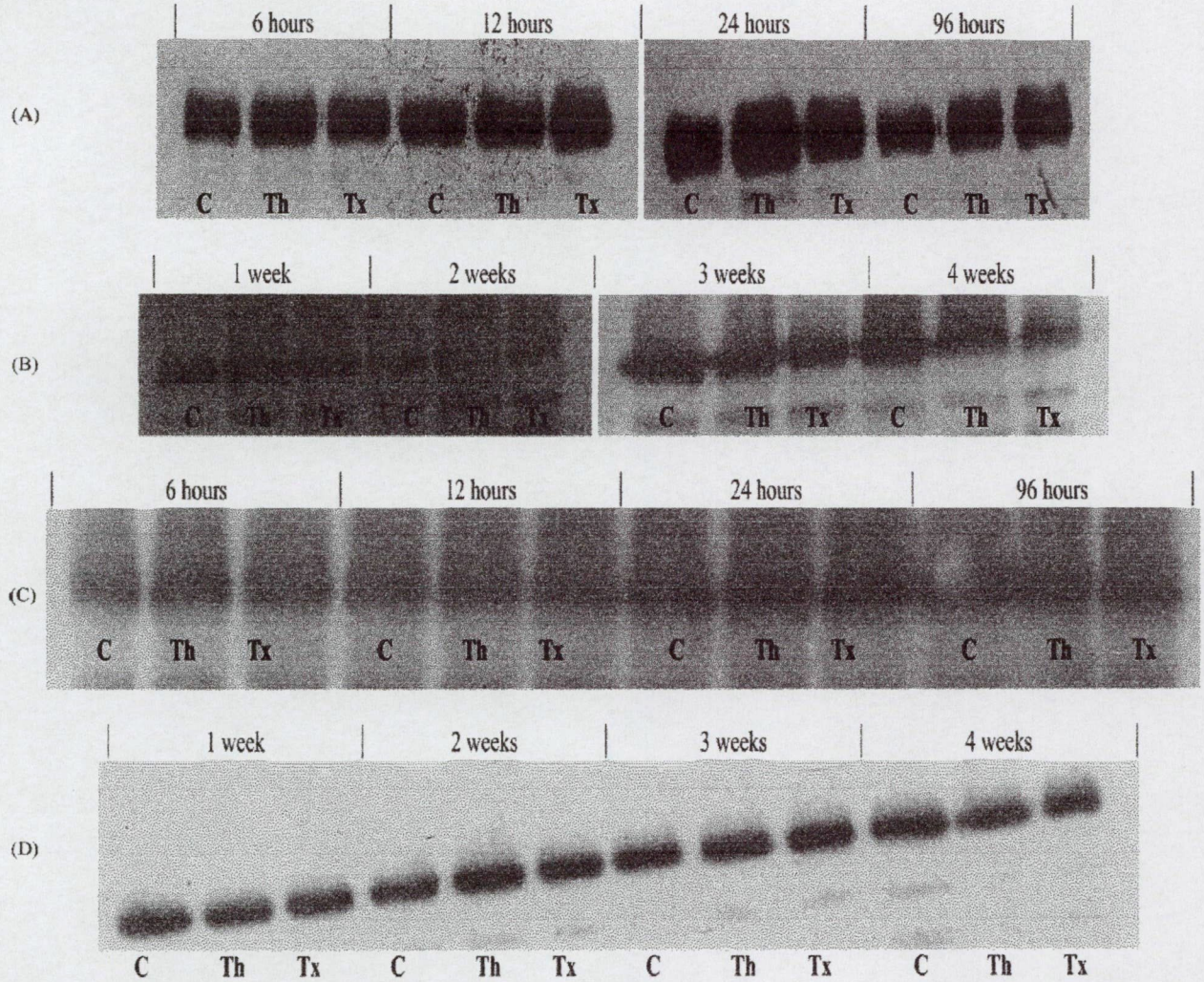

Fig. 1. APP immunoblots. Quantification of immunoblot analysis by densitometry delineates (A) a marked increase after acute haloperidol treatment. (B–D) Chronic haloperidol, and acute or chronic risperidone treatments, respectively, reveal no significant changes either in therapeutic (Th) or toxic (Tx) doses when compared to that seen with controls (C).

2.2. Preparation of rat cortical samples

After acute (6, 12, 24, and 96 h) and chronic (1, 2, 3, and 4 weeks) administration of the antipsychotics in both therapeutic and toxic doses, rats were decapitated under ether anesthesia. The cerebellum was removed and the temporal cortices were dissected. In order to eliminate post-mortem decay, preparation was performed on dry ice.

The samples were homogenized in 50 mM of Tris buffer (pH 7.5) containing 0.15 M NaCl, 2 mM phenyl-methyl-sulfonyl-fluoride, 2 mM EDTA, 2 μg/ml leupeptin, 1 μg/ml pepstatin, 1% Nonidet–P-40 and 0.1% sodium deoxycholate by using a glass–teflon potter (1500 rpm, 1 min). The homogenates were centrifuged at 10,000 × *g* for 30 min at 4 °C. The supernatants were used for the detection of total APP. Protein concentrations of samples were determined by the method of Folin as modified by Hess et al. [24].

2.3. Western immunoblot

Proteins (30 μg per lane) were separated on 9% of sodium-dodecyl-sulfate (SDS)-polyacrylamide gel and electroblotted onto nitrocellulose membranes by using the Bio-Rad Mini-Protean II system. After protein quenching with 5% non-fat dry milk in 50 mM of Tris-buffered saline (TBS, pH 7.5) containing 0.2% of Tween-20, the blotted samples were incubated overnight at room temperature in monoclonal 22C11 antibody (5 μg/ml, against residues 68–81 of APP). After being washed (5×), the membranes were incubated with horseradish-peroxidase (HRP)-conjugated anti-mouse-IgG (1:500, Sigma–Aldrich, USA). For the detection of blots, the Renaissance Western Blot Chemiluminescence Reagent (Pierce, USA) was employed, followed by exposure to an autoradiographic film. Optical densities of immunoreactive bands were detected and quantified by means of the NIH-Image Analyser Program (NIH, USA). The levels of APP in the control group were taken as 100%, and changes were calculated with respect to this value (data are given in relative units).

2.4. Data analysis

Results presented are "arithmetic means ± standard deviation" of experiments performed in triplicate. Student's *t*-test was used to determine the significance of observed differences. All differences stated in the text are statistically significant ($P < 0.05$).

3. Results

Using monoclonal 22C11 antibodies against residues 68–81 of APP, we were able to detect APP-containing wide bands at 110–120 kDa (Fig. 1).

Traditional antipsychotic HAL efficiently elevated cortical APP levels in a concentration-dependent manner (Figs. 1A, B and 2). Significant rise in APP was evident as a result of acute administration of HAL in therapeutic and toxic doses within 24 and 12 h, respectively. Significant changes in APP concentrations were between 2 and 6% when compared to that seen with control levels.

On the other hand, chronic HAL treatment slightly increased APP concentrations, however, did not change APP levels considerably.

Risperidone slightly, but not significantly increased cortical APP levels in both therapeutic and toxic doses during all treatment periods (Figs. 1C, D and 3). In case of this atypical antipsychotic drug there was neither time-, nor concentration-dependence with respect to APP levels.

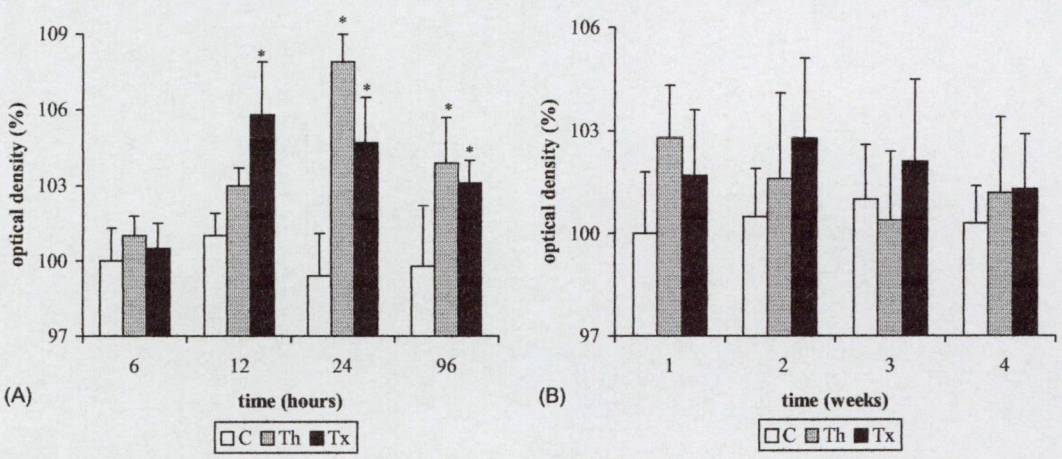

Fig. 2. Semi-quantitative evaluation of cortical APP-Western blot after or without intraperitoneal haloperidol administration. Rat cortical APP levels were approximated utilizing Western blotting, and chemiluminescent visualization of bands were quantified by densitometry. (A) Haloperidol treatment revealed significant increase in both therapeutic (Th) and toxic (Tx) doses within 24 and 12 h, respectively, with regards to APP when compared to that seen with control (C) animals (*$P < 0.05$). (B) Chronic HAL administration yielded slight increase, but no significant changes in APP concentration.

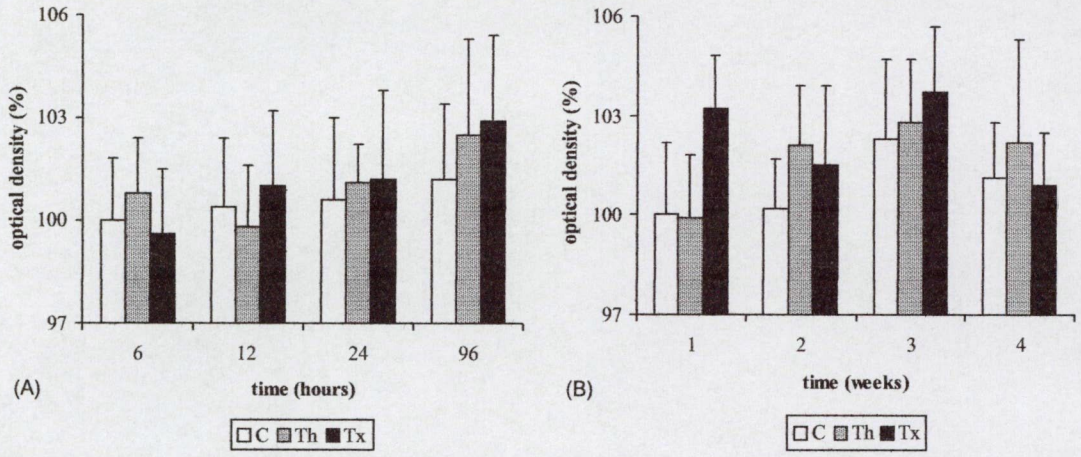

Fig. 3. Time-course of risperidone on rat cortical APP. APP-containing bands were assessed by chemiluminescent Western immunoblot techniques. (A) Acute (6, 12, 24, and 96 h) risperidone treatment, administered through the intraperitoneal route, resulted in incessant APP levels in SPRD rat cortices. (B) Chronic (1–4 weeks) intraperitoneal risperidone treatment does not significantly alter cortical APP in vivo as detected by chemiluminescent Western blotting (C: control; Th, Tx: therapeutic and toxic risperidone treatment, respectively).

4. Discussion

4.1. Effect of HAL on APP levels

This is the first published report on the effect of HAL and RISP on APP metabolism in vivo. Acute administration of HAL apparently yielded APP elevation in both therapeutic and toxic doses in our experiments. Because HAL was demonstrated to act as an inhibitor of select proteinases [16], including secretases that cleave APP [25], acute HAL leads to increase in the non-metabolized form of APP. This is supported by the finding that APP metabolism is increased by environmental signals that activate Ca^{2+}-signaling pathways [31]; however, HAL is a calcium antagonist [43] and therefore it efficiently inhibits APP processing, leading to elevated full-length APP levels.

4.1.1. Neurotoxicity and neuroprotection

HAL has been proved to be neurotoxic, and the involvement of oxidative free radicals and apoptotic factors is strongly implicated [3,5].

σ_1-ligands, such as neurosteroids, play a role in neuroprotection [21,41]. σ_1-agonists modulate ionotropic, metabotropic, and voltage gated Ca^{2+}-signals, and enhance intracellular Ca^{2+}-mobilization [46]. Ca^{2+} increases APP metabolism in general [31], and also stimulates sAPPα secretion [14]. sAPPα can reduce neuronal cytosolic Ca^{2+}-levels and have demonstrated neuroprotective efficacy against a variety of insults [33]. Moreover, σ_1-receptor agonists play an important role in learning and memory. They have been demonstrated to possess neuromodulatory effects; they potentiate cholinergic neurotransmission and the glutamatergic system [29,35]. Also, neuroprotection against βAP-induced neurotoxicity was reported [11]. Because the traditional neuroleptic HAL also acts as sigma σ_1-antagonist [47], it inhibits this complex neuroprotective pathway.

By being a D_2-receptor antagonist [22], HAL decreases PKC activity and level [19]. Activating PKC would lead to secretion of neuroprotective sAPPα [9,13], and modulation of Ca^{2+}-currents by regulating Ca^{2+}-channels through second messengers [38,45]. Ca^{2+}, one of the most important second messengers in the brain, decreases APP mRNA levels [49]. Taken together, decreased PKC by HAL increases APP mRNA.

Even though increased APP level is a precondition to excessive neurotoxic βAP formation, one of the inducible neuroactive (trophic) factors at site of neural injury is the APP that may participate in cell-to-cell recognition, reestablishment of synaptic contacts, alleviation of glutamate-induced membrane depolarization and over-excitation, and stabilization of the intracellular Ca^{2+} homeostasis [28,30,40,41]. In addition, persistent induction of APP expression without subsequent accumulation of βAP in response to acute brain injury substantiates the neuroprotective potential of APP [26,28]. This may account for the elevated APP level seen in our experiments after HAL treatment.

Moreover, HAL increases the activity of NF-κB [42]. Activation of NF-κB by either sAPPα [4] or HAL stabilizes cellular Ca^{2+} homeostasis by modulating the expression of genes that encode calcium-binding proteins, ionotropic glutamate receptor subunits, and anti-apoptotic bcl-2 family members [6,32]. This is in line with our previous experiences with HAL-induced cellular protection with respect to neurotoxic APP metabolites [39].

4.1.2. HAL and Ca^{2+} in AD

Abnormal signal transduction systems have been implicated in the pathophysiology of AD and in APP metabolism, but their precise role has been difficult to establish. We hypothesize that the action of HAL on APP processing is a fine-tuning mechanism involving various second messengers, such as Ca^{2+}.

Calcium, as discussed above, plays a central role in HAL-induced neurotoxicity and neuroprotection both in general and in AD. However, HAL modulates extracellular Ca^{2+}-influx and intracellular Ca^{2+}-mobilization through σ_1-receptors, therefore stabilizes Ca^{2+}. This fine-tuning mechanism leads to constitutive sAPPα secretion, which precludes βAP formation. This is in line with our finding that HAL attenuates βAP-induced Ca^{2+}-imbalance and stabilizes $[Ca^{2+}]_i$ [39]. Moreover, because it has been shown to non-selectively block Ca^{2+}-channels [43], HAL may also directly protect in AD against pores formed in the membrane by βAP that act as cation (e.g. Ca^{2+}) channels [1].

Yielding an elevated APP concentration in the aforementioned calcium-dependent manner by HAL is two-fold: suppressing its cleavage or elevating $mRNA_{APP}$-level. However, HAL has been reported to block the amyloidogenic processing of APP, which, in turn, precludes βAP production [25], and therefore may stimulate neuroprotective sAPPα secretion which may attenuate both self-induced (i.e. direct toxicity of HAL) and βAP-derived neurotoxicity.

Moreover, chronic treatment with HAL results in stable APP levels in our experiments, which leads to decreased deposition of βAP in the brain over time by inhibiting βAP formation [25] and alleviating βAP-induced toxicity [39]. This may explain why several investigators have found a low frequency of AD neuropathology in SCH, as many patients are likely to have been treated chronically with antipsychotics, including HAL, a commonly prescribed drug for this disorder.

4.1.3. APP-receptor

Various ligand-operated ion channels that control intracellular calcium levels may also regulate APP processing [31]. Both sAPPα and βAP have been demonstrated to modulate cytoplasmic Ca^{2+} concentration [33,34], raising the possibility that these compounds may regulate their own formation. This fine-tuning mechanism may be characteristic of AD because of the involvement of various APP-related substances. However, by being a putative cell-surface receptor, APP itself might also be directly involved in this scenario [27]. APP might be a single metabotropic receptor coupled to GTP-binding proteins [36], or it may be a part of a larger receptor complex potentiating D_2- and/or σ_1-receptors. Elevated APP levels after acute HAL treatment argue for this hypothesis, in that inhibitory ligands (e.g. HAL) result in compensatory up-regulation of their receptors (e.g. APP itself). Therefore, HAL-APP receptor-kinetics should be investigated in the future.

4.2. Effect of RISP on APP levels

Atypical RISP has common action with HAL on D_2-receptors. However, it also acts as a serotonergic-antagonist, of which 5-HT_2 and $5HT_4$-receptors have been shown to stimulate sAPPα secretion [37]. Moreover, lesions of cholinergic, serotonergic, and adrenergic neurotransmitter systems all result in the induction of APP levels [48]. By antagonizing both 5-HT and NA (i.e. adrenergic) receptors, slight elevation in APP concentration elicited by RISP treatment is apparently evident. This suggests that RISP may induce neuroprotection against various internal or external nocuous stimuli by slightly stimulating APP expression [26, 28,30].

In contrast with HAL, high potential atypical antipsychotic RISP did not have a significant effect on APP, nor does the literature have any information on its importance at any level in AD, be it clinical or molecular. The observed changes elicited by HAL in this study are therefore better explained. Even though both drugs act as D_2-antagonists, involvement of σ_1-receptors, altered affinity to APP, or other unknown mechanisms may account for the differing impact of HAL from RISP on APP metabolism. However, the slight increase in APP levels as a result of RISP treatment (Fig. 3) might also be regulated by gordian mechanisms; apart from D_2-receptors, the involvement of numerous factors beyond the first messenger level along with 5-HT_2 and NA-receptors might be implicated.

5. Conclusion, future directions

It is feasible that resident brain neurons may acutely have an increased sensitivity to the APP stimulatory action of HAL. However, elevation in the level of the evolutionarily highly conserved APP by any internal or external stimulus would be of enormous functional importance; this is consistent with the observed significant elevation being "only" 2–6% in our experiments. Therefore, the rise in APP levels might be attributable to simple and transient translocation of the precursor with intact expression, and/or altered metabolism to alleviate drug-induced neurotoxicity and promote survival. Because HAL interferes with APP processing in a complex way, the latter appears to be more conspicuous. This hypothesis is supported by our present finding that either acute or chronic administration of RISP, an atypical antipsychotic with no known impact on APP metabolism, has slightly increased, but did not significantly interfere with APP levels in vivo.

When present findings are taken together with their clinical relevance, we reason that both HAL and RISP are considered relatively safe with respect to APP when administered in the treatment of the frequently occuring BPSD in AD. However, in order to examine the effect of both HAL and RISP on APP processing, selective detection of sAPPα should be performed.

The complex agonistic and antagonistic effects of HAL—and probably RISP—on APP metabolism as discussed herewith are not yet understood, but the involvement of Ca^{2+} is clear. This might, however, be co-regulated by various other factors activated or inhibited by these drugs. To address this issue, we aim to establish gene expression profiles of traditional and atypical antipsychotics.

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Haloperidol Attenuates β-Amyloid-Induced Calcium Imbalance in Human Fibroblasts

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Alzheimer's disease · β-Amyloid peptide · Calcium · Fibroblast · Fluorescence · Haloperidol · Schizophrenia

Abstract
Background: Antipsychotics are widely used in the treatment of behavioral and psychological symptoms of dementia. A low frequency of Alzheimer's disease in patients with schizophrenia is reported, and it has been proposed that antipsychotic medications, such as haloperidol, may be responsible. Disruption of intracellular calcium levels is considered to play a key role in β-amyloid-induced neurotoxicity in Alzheimer's disease. Haloperidol has also been reported to interact with calcium homeostasis through dopamine-2 and sigma-1 receptors, and other, yet unknown mechanisms. ***Objective:*** Therefore, we investigated whether differences in the basal intracellular free calcium levels of cultured cutaneous fibroblasts – cells that do not express dopamine-2 and sigma-1 receptors – derived from sporadic Alzheimer patients and from age-matched control individuals after haloperidol treatment might be present. ***Methods:*** Intracellular calcium level was measured in Fura-2AM-loaded human fibroblasts by dual wavelength spectrofluorimetry. ***Results:*** Alzheimer cells exhibited significantly lower calcium level as compared to the control cultures. Exposure of fibroblasts to β-amyloid peptide resulted in increased calcium concentration of the control cells, but not of Alzheimer fibroblasts. Co-incubation of cultures with a therapeutic dose of haloperidol blocked the β-amyloid-induced elevation of calcium. ***Conclusion:*** This finding indicates that haloperidol efficiently countervails ionic imbalance and suggests that it may serve as a potential agent in alleviating neurotoxic effects of β-amyloid peptide.

Introduction

Neurotoxicity caused by the ubiquitously expressed β-amyloid peptide (βAP) is considered to be the underlying pathomechanism in Alzheimer's disease (AD). Accumulation of βAP initiates progressive neuritic injury, which results in disruption of neural metabolic and ionic homeostasis. Destabilization of Ca^{2+} homeostasis in neurons plays a central role in AD pathogenesis [1].

βAP is also reported to form deposits in the skin of AD patients [2]. A large number of studies have showed var-

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ious alterations in fibroblasts, such as disturbances of Ca^{2+} uptake [3], glucose metabolism [4] and several other biochemical changes [5, 6]. In our ongoing studies we have demonstrated alterations in biochemical processes in cultured skin fibroblasts of AD donors. We have proved that, in contrast to control cells, AD fibroblasts exhibit low resting intracellular calcium concentration ($[Ca^{2+}]_i$) and are resistant to βAP.

Several reports demonstrate that AD pathology is infrequent in patients with schizophrenia (SCH) [7, 8]. It has been proposed that medications used to treat SCH, such as haloperidol (HAL), might have a protective effect against developing AD neuropathology, which would account for a low frequency of these changes in elderly patients with SCH. The mechanism by which antipsychotic drugs might exert such an effect is not known. The use of neuroleptic medication in AD has recently been suggested, in that select antipsychotics are widely used in the treatment of behavioral and psychological symptoms of dementia, including psychosis, delusions, hallucinations, agitation, etc. – occurring in roughly half of AD patients.

HAL has previously been reported to interfere with calcium metabolism through various mechanisms, including interaction with dopamine-2 and sigma-1 receptors [9–11]. It has been proposed that other pathways also lead to HAL-induced changes in Ca^{2+} levels. Because fibroblasts do not express dopamine-2 and sigma-1 receptors, and in light of evidence that HAL interferes with Ca^{2+} homeostasis, we evaluated HAL for its effects on βAP-induced Ca^{2+} imbalance on fibroblasts. In the course of surveying a wide variety of agents directed against βAP toxicity, here we report that HAL, one of the most frequently prescribed antipsychotic medications used in the treatment of SCH [12], counterbalances βAP with respect to $[Ca^{2+}]_i$.

Materials and Methods

Patient Population

Forearm excision biopsies were obtained after informed consent from late-onset sporadic AD patients (aged 73 ± 10.11, $n_{AD} = 42$) and age-matched controls (aged 71 ± 8.8, $n_C = 16$). At the time of skin biopsy, all participants were outpatients. The diagnosis of AD was made according to DSM-IV criteria [13]. All AD donors met the DSM-IV definition of dementia of the Alzheimer type, which requires evidence of cognitive deficits (criterion A) and a process of decline from previous levels (criterion C); none of them had a history of familial AD. Control individuals were tested negative for any forms of dementia.

None of the participants in our study received any medication known to interfere with calcium metabolism, such as antihypertensive drugs, calcium antagonists, or antidepressants. None of the patients was on acetylcholinesterase inhibitor before and during the measurements. No probands suffered from any hematologic or inflammatory disorders.

Fibroblast Culture Methods

All AD and control samples were cultured in the same conditions, as discussed previously [14]. In short, fibroblasts were grown on glass coverslips in a 24-multiwell plate in Dulbecco's modified Eagle's medium containing 5% of heat-inactivated fetal calf serum as well as penicillin (100 U/ml), streptomycin (100 μg/ml) and 2 m*M* glutamate in a humidified atmosphere of 95% air and 5% of CO_2. Cultures used for measurements of $[Ca^{2+}]_i$ were generally confluent. All fibroblasts had a characteristic spindle-shaped appearance and were attached firmly to the coverslips. Viability of cells was estimated by the method of intravital staining with trypan blue after the incubation of cell suspension in the presence of the preparation (0.2% trypan blue in Hanks' balanced saline solution. After 5 min of incubation in the solution, 1.3% of cells absorbed the dye. In line with this finding we concluded that 98.7% of fibroblasts present on the coverslips were viable.

Measurement of Basal Intracellular Free Calcium Levels

$[Ca^{2+}]_i$ values were quantified by fluorescence ratio imaging of the calcium indicator dye Fura-2AM. Cells were incubated for 30 min in the presence of 1 μ*M* of the acetoxymethylester form of Fura-2AM. Cultures loaded with the dye were then washed twice with phosphate-buffered saline and were used immediately for measuring fluorescence intensities. Images were obtained using a Hitachi F-2000 spectrofluorimeter. The ratio of the fluorescence emission of the cultures using two different excitation wavelengths of 340 and 380 nm at emission wavelength of 495 nm was applied to determine $[Ca^{2+}]_i$. In line with many other papers examining calcium levels [2, 14–17], the authors found the rate of the fluorescence ratios excellent for monitoring alteration of $[Ca^{2+}]_i$, without calculating actual $[Ca^{2+}]_i$ values.

To check that labeling of the cells with the dye and intracellular cleavage of the ester occurred, we sampled the fluorescence excitation ratios before loading the cultures with the dye. Ratios of unlabeled cells observed at 495 nm were 12.03 ± 5.04, around 4 times lower when compared to the ratio of 46.11 ± 9.19 after incubation with the dye; this is characteristic of Fura-2AM fluorescence.

No significant alteration was detectable among labeled cultures in Fura-2AM fluorescence using the calcium-insensitive excitation wavelength of 367 nm, indicating that the observed response reflects a real change in the calcium level of the cells.

Because gross alterations in the cell number/cell mass actually present in the cuvettes may interfere with the fluorescence intensities, the total protein content of the cuvettes was determined according to Lowry et al. [18]. Cuvettes were found to contain 142.3 ± 10.1 μg protein/cuvette, with no cuvette significantly deviating from the mean. This suggests that the total protein content of the cultures examined did not differ considerably.

Drugs

β-Amyloid Peptide. Solid-phase protein synthesis was used for the preparation of $βAP_{1-42}$ [19], as discussed previously [16]. Peptides were purified by a Shimadzu LC-8A preparative HPLC system. Pure

βAPs were dissolved in phosphate-buffered saline and were incubated for an hour to 'age' before direct use in the cultures. In the experiments where βAP was used, fibroblasts were incubated in 10^{-7} *M* final concentration of the peptide for 16 h. This concentration of βAP is equivalent to that seen in the plasma of AD patients. Control cultures were free of βAP treatment.

Haloperidol. The antipsychotic Haloperidol® was obtained commercially from Gedeon Richter Ltd. and was used directly in the experiments. Final HAL concentration was 10 ng/ml, which equals to the therapeutic plasma level of HAL [20, 21]. Control cultures were free of HAL treatment.

Data Analysis

Values are given as arithmetic mean ± standard deviation. All differences stated in the text are statistically significant (Student's t tests, $p < 0.05$).

Results and Discussion

Fibroblasts were successfully harvested from both AD and control donors. Cells were competent to be labeled with Fura-2AM at 37°C by passive diffusion during the experiments. In case of control specimens, the 340/380 nm fluorescence excitation ratio (FL), representing $[Ca^{2+}]_i$, revealed 2.48 ± 0.162, whereas AD samples exhibited an FL of 2.052 ± 0.207 (table 1). Control fibroblasts incubated for 16 h in 10^{-7} *M* of fresh βAP demonstrated a significant increase in $[Ca^{2+}]_i$ (FL 2.666 ± 0.08). In contrast, AD cultures, maintained in the same condition, showed little or no change in the free cell calcium (FL 2.055 ± 0.125). $[Ca^{2+}]_i$ of neither control nor AD fibroblasts have changed significantly as a result of HAL treatment (FL 2.41 ± 0.119 and 2.049 ± 0.202, respectively). Preincubation of cultures with HAL together with βAP has also resulted in no elevation in $[Ca^{2+}]_i$ (table 1, fig. 1).

These findings indicate that cultured cells from donors of AD demonstrate significant decreases in free cell calcium when compared to that of age-matched controls. These data clearly indicate that detectable biochemical alterations are present in Alzheimer fibroblasts.

Our data also implies that chronic exposure of cells to βAP causes a rise in free cell calcium only in control fibroblast cultures. βAP disrupts calcium-regulating processes in control cells resulting in elevations of free cell calcium. AD fibroblasts do not show this change. A substantial body of data exists indicating that AD fibroblasts are rendered resistant to βAP as a result of the long-term exposition of the peptide. Taken together with our previous and ongoing experiments, we hypothesize that tonic βAP load ultimately decreases $[Ca^{2+}]_i$ and makes cells insensitive to β-amyloid in the long run.

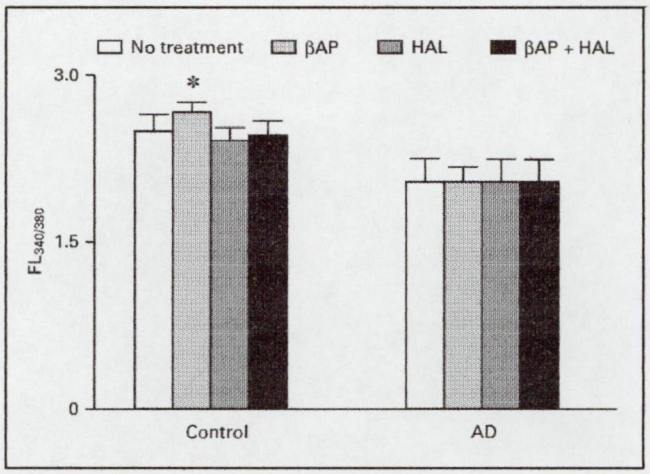

Fig. 1. 340/380 nm fluorescence excitation ratios. The chart delineates that control fibroblasts demonstrate increases in the basal intracellular Ca^{2+} level as a result of βAP treatment. Co-treatment of cultures with HAL attenuates βAP-induced calcium imbalance. Basal Ca^{2+} concentration of AD cells is significantly decreased when compared to that of the controls. AD fibroblasts have proved to be resistant to both βAP and HAL with respect to calcium. * $p < 0.05$.

Table 1. 340/380 nm fluorescence excitation ratios ($FL_{340/380}$)

Cultures	$FL_{340/380}$ (mean ± SD)	Coverslips
Control	2.48±0.162	16
AD	2.052±0.207	42
Control + βAP	2.666±0.08	16
AD + βAP	2.055±0.125	42
Control + HAL	2.41±0.119	16
AD + HAL	2.049±0.202	16
Control + βAP + HAL	2.46±0.123	16
AD + βAP + HAL	2.054±0.198	16

Comparative fluorimetric studies on human control and AD fibroblasts on the $FL_{340/380}$ using the Ca^{2+} indicator dye Fura-2AM. The cells were cultured with or without 10^{-7} *M* of βAP and/or 10 ng/ml of HAL for 16 h at 37°C. FL were calculated from fluorescence intensities observed at 495 nm using excitation wavelengths of 340 and 380 nm.

HAL, a calcium and calmodulin antagonist [11], neutralized βAP-induced $[Ca^{2+}]_i$ elevation in our experiments. Piling evidence suggests that HAL blocks Ca^{2+} channels [22, 23]. Also, a substantial body of published data has established that βAP can form ion channels in lipid bilayers, liposomes, neurons and other cells [24, 25].

βAP channels are heterogeneous in size, selectivity, blockade, and gating. They exhibit multiple cation selectivity, admitting Ca^{2+}, Na^{+}, K^{+}, Li^{+}, etc., leading to unregulated calcium influx via βAP channels. Based on our present findings and on the Ca^{2+} channel hypothesis of βAP, we propose that HAL may efficiently block the channel activity of βAP, too.

Because HAL was demonstrated to efficiently inhibit βAP formation [26], we hypothesize that the protective effect of HAL is achieved through two associated mechanisms. It might decrease the concentration of βAP; accordingly, less Ca^{2+} channels, formed of βAP, will be present; however, even these few channels will be blocked by HAL. Our results strongly support the hypothesis that both regulating βAP production [26] and blocking βAP channels may be underlying the molecular mechanism of HAL (neuro)protection.

A growing number of evidence indicates that HAL interacts with calcium homeostasis. Apart from directly modulating calcium levels and blocking Ca^{2+} channels, HAL also alters the expression of genes involved in the regulation of $[Ca^{2+}]_i$ of neurons [27]. Moreover, HAL has been reported to induce apoptosis of brain cells, which involves activation of sigma receptors, and also translocation of calcium into the cytoplasm [9, 10]. On the contrary, however, our results suggest that HAL itself does not change Ca^{2+} level in fibroblasts. To the best of the authors' knowledge, this is the first published data on the effect of HAL on fibroblasts regarding $[Ca^{2+}]_i$.

Because of differences certain to be encountered with respect to tissue-specific macromolecules, lack of various neuronal receptors on fibroblasts – including sigma and dopamine receptors – may give an explanation. Nevertheless, HAL efficiently attenuated βAP-induced calcium imbalance in our experiments. In spite of its inability to interfere with Ca^{2+} in the periphery, this observation further confirms the possibility that HAL directly interacts with βAP. We hypothesize that it either operates as a β-sheet breaker, or inhibits βAP channel formation and/or βAP channel activity. However, further studies are needed to evaluate the putative neuroprotective effect of HAL. These might include selective pre- and postincubation of cultures with HAL and βAP; comparing our experiences with fibroblasts with cultures from central nervous system that do (e.g. neurons) and those that do not (e.g. glias) express dopamine and sigma receptors, and biophysical studies on the interaction between HAL and βAP.

Chronic treatment with HAL may result in decreased βAP-induced neurotoxicity in the brain over time through various, but not fully understood mechanisms as discussed above. This may explain why several investigators have found a low frequency of AD neuropathology in SCH, as many patients with SCH are likely to have been chronically treated with HAL, a commonly prescribed drug for this disorder.

Because AD is frequently associated with behavioral and psychological symptoms of dementia, which is treated with antipsychotic medications such as HAL [28, 29], future randomized controlled studies should target AD patients with behavioral and psychological symptoms of dementia with a history of chronic HAL treatment for neuropathologic examination. Our prediction is that these patients should show a lower progression rate than untreated subjects or subjects treated with structurally divergent antipsychotic drugs. In light of evidence, to this end, HAL may be a useful lead in the development of an effective AD therapeutic agent.

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