Lithium Decreases Secretion of A β 1–42 and C-Truncated Species A β 1–37/38/39/40 in Chicken Telencephalic Cultures but Specifically Increases Intracellular A β 1–38

H. Esselmann^a J.M. Maler^c N. Kunz^a M. Otto^b S. Paul^c P. Lewczuk^c
E. Rther^a J. Kornhuber^c J. Wiltfang^c

Departments of ^aPsychiatry and ^bNeurology, University of Gttingen, Gttingen, and
^cDepartment of Psychiatry and Psychotherapy, University of Erlangen-Nrnberg, Erlangen, Germany

Key Words

Telencephalic neurons, chicken · β -Amyloid ·
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Abstract

We studied endogenous amyloid precursor protein (APP) processing and amyloid beta (A β) peptide formation in primary chicken telencephalic neurons, because their A β peptide sequence is identical to humans. As detected by quantitative A β -SDS-PAGE/immunoblot, A β peptides 1–40/42 and three additional C-truncated species, namely A β 1–37/38/39 were regularly released into the supernatant. The highly conserved A β quintet strongly resembles the pattern of A β peptides found in human cerebrospinal fluid. Furthermore, the C-terminally shorter A β peptides 1–33/34 could be readily detected. Recent evidence indicates that lithium specifically inhibits secretion of the amyloidogenic A β 1–42 peptide in cultured permanent cells transfected with human APP. We therefore investigated the effect of lithium on A β peptide secretion as well as intracellular A β peptides in our untransfected primary cell culture system. Our data shows that lithium leads to a dose-dependent reduction of A β 1–37/38/39/40/42 secretion. Surprisingly, intracellular analysis revealed that lithium specifically increases a

band comigrating with synthetic A β 1–38 while A β 1–40 and A β 1–42 remained almost unaffected. These results demonstrate for the first time that lithium treatment decreases A β peptide secretion in primary chicken neuronal cells but specifically elevates intracellular A β 1–38. Therefore, we conclude that there are two independent mechanisms of lithium in intra- and extracellular A β peptide production.

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Introduction

The amyloid precursor protein (APP) has been intensively studied since its discovery in 1987 because its proteolytic processing leads to β -amyloid peptide (A β peptide) formation [1]. Potentially neurotoxic A β peptides play an important pathophysiological role in Alzheimer's disease (AD) as they aggregate forming neuritic plaques [2].

Whereas cleavage of the transmembrane APP by α -secretase results in a nonamyloidogenic metabolic pathway and release of secreted APP (sAPP α), β - and γ -secretase cuts lead to generation of A β peptides [3]. Several A β peptide variants are known, of which A β 1–40 is the predominant one in human cerebrospinal fluid (CSF) and

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Prof. Dr. J. Wiltfang, Molecular Neurobiology Laboratory
Department of Psychiatry and Psychotherapy, University of Erlangen-Nrnberg
Schwabachanlage 6, DE-91054 Erlangen (Germany)
Tel. +49 9131 8536896, Fax +49 9131 8536381
E-Mail Jens.Wiltfang@psych.imed.uni-erlangen.de

A β 1–42 the species initially deposited in AD β -amyloid plaques [4]. A β peptides 1–40 and 1–42 are thought to be derived by the action of different γ -secretase activities [5]. Additionally, a set of carboxyterminally truncated A β peptides termed 1–37/38/39 in a highly conserved pattern has recently been identified in human CSF [6]. The use of this A β peptide quintet pattern proved to be useful in the differential diagnosis of patients with AD compared to those with chronic inflammatory CNS disease or other neuropsychiatric diseases.

Recently it has been demonstrated that lithium specifically inhibits the secretion of the amyloidogenic A β 1–42/40 in cell lines transfected with human APP [7, 8]. This suggests that lithium may be implicated in A β peptide metabolism and deposition. Therefore, we studied the effect of lithium on the A β peptide secretion pattern and intracellular A β peptide of chicken telencephalic neurons. In our study, a primary neuronal cell culture model derived from the chicken telencephalon was used, because the chicken A β region exhibits sequence identity with the human A β peptide [9, 10].

The present study was undertaken to investigate the A β secretion characteristics of chicken telencephalic neuronal cultures and its pharmacological modulation by lithium. A β peptides were analyzed by attomolar-sensitive A β -SDS-PAGE/immunoblot.

Materials and Methods

Animals and Materials

Fertilized White Leghorn chicken eggs were obtained from the institutional animal care facility. All procedures using animals were approved by the Institutional Animal Care and Use Committee. Synthetic A β peptides 1–38/40/42 were obtained from Bachem. Other synthetic A β peptides were synthesized automatically using the Fmoc chemistry according to Janek et al. [11] and were kindly provided by M. Beyermann, Research Institute of Molecular Pharmacology, Berlin, Germany. LiCl and NaCl were from Sigma (Germany).

Culture of Chicken Telencephalic Neurons

Telencephalic cortices were dissected from 10-day-old chicken embryos and cultured according to Pettmann et al. [12]. Briefly, after removing the meninges, telencephalic hemispheres were digested by incubation with trypsin/EDTA solution (0.05/0.02%) for 15 min at 37°C and then dissociated by aspiration through a 20-gauge needle. The homogenate was centrifuged (550 g, 5 min) and resuspended into culture medium consisting of Dulbecco's modification of Eagle's medium supplemented with 5% fetal calf serum and 5% chicken serum. To remove undissociated tissue the suspension was passed through a sterile nylon sieve (80 μ m) and cells were finally seeded at a density of $3.75 \cdot 10^5$ cells/cm² onto polylysine-coated 24-well culture plates. After 30 min, following cellular attachment, incubation medium was renewed. Cultures were grown

at 37°C in a humidified atmosphere of 95% air and 5% CO₂, the nutrient medium was changed after 3 days and experiments were performed between the 5th and 6th day in vitro. Chicken serum was obtained from Gibco; all other cell culture reagents were purchased from Biochrom.

Assessment of Cell Viability

Cell viability was assessed morphologically and quantified using the CytoTox 96® lactate dehydrogenase assay kit (Promega) and the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, Sigma] assay according to Mosmann [13].

Processing of Cells and Supernatants for A β Peptide Assessment

After an incubation period of 24 h, supernatants of 4 separate wells exposed to the same experimental conditions were pooled, spun at 14,000 g for 5 min and immediately frozen at –80°C. Cells (seeding density $9 \cdot 10^6$) were washed twice in PBS, scraped from their supports, spun at 3,000 g (5 min, 4°C), snap frozen in liquid nitrogen and stored at –80°C until further use. Lysis was performed in RIPA buffer (30 min, on ice). Insoluble material was spun down at 14,000 g (5 min, 4°C) and the supernatant used for intracellular analysis. The protein content was determined with the BCA kit (Pierce) using BSA as standard.

A β -SDS-PAGE/Immunoblot

For the separation and visualization of A β peptides from cell lysates and supernatants the urea version of the bicine/sulfate SDS-PAGE and the semidry Western blotting method according to Wiltfang and colleagues [4, 6, 14] were used. The SDS-PAGE is based on the bicine/bis/tris/sulfate multiphasic buffer system with 8 M urea separation gels, which was used by Wiltfang et al. [15] and Klafki et al. [14] for the first time without further modification for the separation of A β peptides. Briefly, following concentration of A β peptides by immunoprecipitation and addition of sample buffer, samples were transferred to gel slots. Gels were run at room temperature for 2 h at a constant current of 12 mA/gel using the MiniProtein II electrophoresis unit (BioRad). After the blotting step onto PVDF membranes, immunostaining was performed with the monoclonal amino-terminal-selective antibody 1E8 (Nanotools). Next, membranes were washed and incubated with an anti-mouse biotinylated antibody (Vector Laboratories). Following a further washing step, horseradish peroxidase-coupled streptavidin was added for 1 h and chemiluminescence visualized with ECL^{Plus} solution (Amersham Pharmacia) according to the manufacturer's procedure, using the Fluor-S Max MultiImager. Immunoreactivity band intensities were quantified with the Quantity One v4.1 software (BioRad). All samples were run as quadruplicates and each gel carried a five-step dilution series of a synthetic A β peptide mix. The inter- and intra-assay coefficients were below 10%. Mean values were used for subsequent calculations.

Detection of Full Length APP and CTF β (C99)

For APP full length analysis equal amounts of lysed cell protein were mixed with 2 $\times$ sample buffer and boiled for 5 min at 95°C. Samples with equal amounts of protein were transferred to 10% Laemmli gels. After blotting onto PVDF membranes, immunostaining was performed with the 1E8 antibody (22C11 antibody directed against the N-terminus of APP showed the same results – data not shown). The further visualization was the same as described above. For analysis of CTF β (C99) cell lysates were immunoprecipitated

Fig. 1. Representative Aβ-SDS-PAGE/immunoblot of chicken neuronal cell culture supernatants. Aβ peptides from supernatants collected between the 5th and 6th day in vitro were immunoprecipitated with the monoclonal antibody 1E8. Samples were separated on 8 M urea 12%T5%C gels, transferred to PVDF membranes and immunostained with the 1E8 antibody (right panel). A characteristic quintet pattern of Aβ peptides 1–37/38/39/40/42 can clearly be observed. Additionally, an immunoreactivity band comigrating with synthetic

Aβ1–33/34 is detectable (*). ** = Synthetic Aβ1–35 (middle panel). Band intensities of 4 blots per experiment were quantified using a molecular imager with image analysis software (Quantity One) and averaged. The amount of each Aβ species relative to Aβ total is highly stable (table on the right). For quantification a serial dilution of a synthetic standard mix of Aβ peptides (1–37/38/39/40/42/2–42) was applied to every gel (left panel). CN sup = Chicken neuronal cell culture supernatant.

with the 1E8 antibody and separated on 8 M urea 12%T5%C gels, transferred to PVDF membranes, and immunostained using the monoclonal 1E8 antibody. Visualization were the same as described above.

Results

A Highly Conserved Pattern of Aβ Peptides in Chicken Neuronal Conditioned Media

Aβ peptides were secreted by chicken telencephalic neurons in a time-dependent manner. Aβ peptides 1–40 and 1–42 as well as three additional C-truncated species, namely Aβ peptides 1–37, 1–38, 1–39, were regularly expressed (fig. 1). This quintet pattern of Aβ peptides was highly conserved and showed the following order of relative abundance (% , mean ± SD): Aβ1–40: 55.4 ± 6.4, Aβ1–38: 14.7 ± 1.6, Aβ1–42: 8.3 ± 1.2, Aβ1–37: 11.9 ± 1.3, and Aβ1–39: 9.5 ± 1.7. The total amount of Aβ peptides was 671 ± 127 pg/ml. Furthermore, an immunoreactive band was identified, which comigrated with synthetic Aβ1–33 and Aβ1–34 (fig. 1). An incubation of conditioned supernatant on cell-free culture plates showed no qualitative and only minor quantitative changes in the Aβ profile (data not shown). The total Aβ content of the medium, which included 10% serum, was only about

10 pg/ml. Thus, a proteolytic processing of secreted Aβ peptides by medium components and an influence of Aβ peptides originating from chicken and fetal calf serum were negligible.

Modulation of the Aβ Peptide Secretion by Lithium

Incubation of primary chicken telencephalic neurons with lithium (24 h) dose-dependently induced a significant decrease of secreted Aβ1–42 and of C-truncated Aβ1–40/39/38/37 while NaCl (20 mM) had no effect (fig. 2a). The decrease of Aβ secretion observed did not only affect Aβ1–42 but also Aβ1–40/39/38/37 to the same extent (fig. 2b, c).

Modulation of Intracellular Aβ Peptide, APP and CTFβ (C99) by Lithium

The effect of lithium (10 mM) on the RIPA-soluble intracellular Aβ peptide pattern was different in comparison to extracellular secretion as no significant change was observed for Aβ1–42/40. Surprisingly, intracellular Aβ1–38 was slightly increased (fig. 3a). Full length APP and CTFβ (C99) expression remained unaffected by lithium treatment (fig. 3b, c). This indicates that the effect on Aβ secretion was not due to a general suppression of APP synthesis or an inhibition of the γ-secretase pathway.

Fig. 2. Dose-dependent effect of lithium on A β peptide secretion by chicken telencephalic neurons. Lithium dose-dependently induced a significant decrease of secreted A β 1–42 and C-truncated A β 1–40/39/38/37 while NaCl (20 mM) treatment had no effect (**a**). Supernatants (24 h) of untreated or treated cells were collected, centrifuged and A β peptides were concentrated by immunoprecipitation with the monoclonal antibody 1E8. Samples were separated on 8 M urea 12%T5%C gels, transferred to PVDF membranes and immunostained using the monoclonal 1E8 antibody (**a**). Band intensities of 4 blots per experiment were quantified using the Fluor-S Max molecular imager and Quantity One image analysis software. Experiments were performed 4–10 times with similar results. Data are expressed as ng/ml A β peptides (**b**) or as percentage of untreated control, set at 100% (**c**), and represent means $\pm$ SD of 4–10 separate experiments and cultures. A β peptides and bars are aligned from left to right in the order: A β 1–42/40/39/38/37 and total A β (**b**, **c**).

Fig. 3. Effect of lithium on intracellular A β peptide level, full length APP and CTF β (C99). Neuronal cultures were exposed to lithium (10 mM, 24 h), scraped in ice-cold PBS, centrifuged at 3,000 g, snap frozen in liquid nitrogen and stored at -80°C . Cells were lysed in RIPA detergent buffer on ice for 30 min, followed by centrifugation at 14,000 g. Insoluble material was discarded and immunoprecipitated homogenate was used for intracellular analysis of A β peptides (**a**) and CTF β (C99) (**b**) and separated on 8 M urea 12%T5%C gels, transferred to PVDF membranes and immunostained using the monoclonal 1E8 antibody. For analysis of intracellular full length APP (**c**), equal amounts of protein (homogenate) were separated on 10% Laemmli gels, transferred to PVDF membranes and immunostained with the 1E8 monoclonal antibody (22C11 antibody directed against the N-terminus of APP showed the same results – data not shown). Data shown are representative of at least four independent experiments with similar results.

Discussion

Targeting the deposition of amyloid into neuritic plaques has been suggested as a pharmacological approach to alter the progression of AD [16, 17]. To address this question in an easily accessible cell culture system, we used chicken telencephalic neurons. In contrast to rodent cells they share A β sequence identity with man and do not have the disadvantage of a quantitatively or qualitatively different APP metabolism found in many primary non-neuronal human cell culture models and in human cell lines [18–20].

Primary chicken telencephalic cultures secreted A β peptides in the same quintet pattern – consisting of A β 1–37/38/39/40/42 – that is normally found in human CSF [6]. The relative A β peptide quantities were regulated within a narrow range. These findings, including the relative abundance of each A β peptide species, were confirmed by SELDI-TOF MS [21] (unpubl. data).

We further observed that the secretion of A β 1–42 and C-truncated A β 1–40/39/38/37 in chicken telencephalic neurons was significantly reduced by lithium. These results confirm a recent finding that lithium reduces A β 1–40/42 production in Chinese hamster ovary cells stably

expressing APP695 and primary cultures of mouse embryonic cortical neurons infected with Semliki forest virus encoding wild-type APP or the pathogenic APP-Swedish mutation [7] as well as COS7 cells transfected with APP C100 [8]. Additionally, Phiel et al. [7] could show that lithium, a glycogen synthase kinase 3 (GSK-3) inhibitor [22], inhibits the generation of A β peptides through inhibition of GSK-3 α . Presenilin also interacts with GSK-3 [23, 24], but a functional role for this protein in γ -secretase activity has not been established yet. As total amounts of intracellular full length APP and CTF β (C99) remained constant in our cellular model in contrast to the findings of Phiel et al., where an accumulation of CTF β (C99) was observed after lithium treatment, this might reflect differences in APP processing between the intracellular and the secreted pool of A β peptides.

In conclusion, we demonstrated that cell cultures of chicken telencephalic neurons represent a suitable model to study A β peptide metabolism and secretion. The highly conserved A β peptide pattern in supernatants of this cell culture system closely resembles the profile found in human CSF and it is qualitatively regulated in a narrow range. In this model system lithium selectively reduces secreted A β 1–42 and C-truncated A β 1–37/38/39/40. Sur-

prisingly, lithium specifically increased intracellular A β 1–38 without altering intracellular A β 1–42/40 or CTF β (C99). Therefore, we postulate two independent mechanisms of lithium in intra- and extracellular A β formation. Further studies are needed to clarify the role of lithium in A β metabolism in our model system.

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