

Memantine treatment decreases levels of secreted Alzheimer's amyloid precursor protein (APP) and amyloid beta (A β) peptide in the human neuroblastoma cells

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ARTICLE INFO

Article history:

Received 22 September 2009

Received in revised form 4 November 2009

Accepted 5 November 2009

Keywords:

Amyloid
Alzheimer's drug
beta-Peptide
Dementia
Neuronal cells
Protein processing
sAPP
Secretase
Tissue culture

ABSTRACT

Memantine, an uncompetitive NMDA receptor antagonist, is a FDA-approved drug used for the treatment of moderate-to-severe Alzheimer's disease (AD). Several studies have documented protective roles of memantine against amyloid beta (A β) peptide-mediated damage to neurons in both *in vitro* and *in vivo* models. Memantine is also effective in reducing amyloid burden in the brain of APP transgenic mice. However, the exact mechanism by which memantine provides protection against A β -mediated neurodegenerative cascade, including APP metabolism, remains to be elucidated. Herein, we investigated the effect of memantine on levels of the secreted form of A β precursor protein (APP), secreted A β and cell viability markers under short/acute conditions. We treated neuronal SK-N-SH cells with 10 μ M memantine and measured levels of secreted total APP (sAPP), APP α isoform and A β _(1–40) in a time dependent manner for up to 24 h. Memantine significantly decreased the levels of the secreted form of sAPP, sAPP α and A β _(1–40) compared to vehicle treated cells. This change started as early as 8 h and continued for up to 24 h of drug treatment. Unlike sAPP, a slight non-significant increase in total intracellular APP level was observed in 24-h treated memantine cells. Taken together, these results suggest a role for memantine in the transport or trafficking of APP molecules away from the site of their proteolytic cleavage by the secretase enzymes. Such a novel property of memantine warrants further study to define its therapeutic utility.

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Alzheimer's disease (AD) is characterized typically by deposition of amyloid beta (A β) peptide-containing plaques in the brain parenchyma and associated neuro-inflammation, and by severe cholinergic neuronal and synapse loss [27]. A β peptides, which primarily consist of a short peptide of 40 amino acids in length A β _(1–40) and a longer and more 'amyloidogenic' peptide of 42 residues A β _(1–42), are proteolytic cleavage products of a transmembrane protein, amyloid beta precursor protein (APP) by the enzymatic action of β and γ secretases [4,18]. APP can also be processed by another enzyme, α secretase, to produce a truncated ectoplasmic APP, termed sAPP α , which is considered to be a product of a 'non-amyloidogenic' pathway [27]. Currently, cholinesterase inhibitors (ChEIs) and the uncompetitive N-methyl-D-aspartate (NMDA) receptor antagonist, memantine, are the only treatment options available for AD [26]. Although the main purpose of ChEIs

is to prevent hydrolysis of the neurotransmitter acetylcholine to augment its levels in brain, potent anti-A β properties have also been discovered for several ChEIs [16]. We have recently shown that posiphen, a newly developed analogue of a ChEI, dose dependently decreased levels of APP and A β in both cell culture and *in vivo* [14]. Memantine is an uncompetitive NMDA receptor antagonist clinically used for the treatment of moderate-to-severe AD. The NMDA receptor plays an important role in synaptic plasticity and neurodegeneration through the Ca⁺⁺ signaling pathway [5]. Reduction in Mg⁺⁺ blockade of the NMDA receptor-channel not only leads to excessive influx of Ca⁺⁺ in neurons, but also may induce downstream activation of Ca⁺⁺ dependent enzymes and protein kinase signaling pathways leading to excitatory damage of neuronal cells [12]. Several studies have revealed remarkably improved spatial learning in transgenic animals, and enhanced cognitive functions in AD patients treated with memantine [21,24]. Although a number of studies have documented the effect of memantine in protecting neuronal cells from A β -mediated damage both in cell culture and animals [7,29], the action of memantine on levels of APP and A β remains to be fully elucidated. In this regard, we have recently reported that memantine (1 μ M) decreases sAPP and A β (1–40) lev-

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els in human SK-N-SH cells and A β (1–42) levels in AD transgenic mice as early as 3–7 days following treatment [1]. In the present study, we treated human SK-N-SH cells with 10 μ M of memantine and measured levels of sAPP, sAPP α , intracellular APP and secreted A β (1–40) over a much shorter time course to provide insight into memantine's role in the processing of the APP molecule.

In several cell culture based studies, memantine exerted pharmacological activity between 1 μ M and 20 μ M doses depending upon the cell types [1,2,23]; we hence selected a single dose of 10 μ M memantine for the current study. Human SK-N-SH cells were grown in 100 mm plates as described previously [20]. Once the cells became 80% confluent, 250,000 cells were transferred to each well of a six-well tissue culture plate (Corning Inc., NY, USA). Memantine (Sigma, MO, USA) was dissolved in low serum (1% FBS)-containing MEM medium at a final concentration of 10 μ M, and 2 ml of the memantine containing medium was added into each of two wells ($n=2$). Similarly, 2 ml of low serum medium without the drug was also added into each of two wells, which served as vehicle. The plate was incubated at 37 °C, and 200 μ l of conditioned media (CM) sample was collected from each well at 8-h intervals up to 1 day (8, 16 and 24 h, respectively). Cells were harvested at the end of 24 h and lysed by 'IP' lysis buffer, as described [17]. Cell viability in each well was determined by the luminescence based Cell Titer Glo (CTG, Promega Corporation, WI, USA) assay. CTG measures ATP concentrations, which reflects the cell number. Cell toxicity within the treatment groups was determined by LDH assay with 30 μ l of CM sample, as described [8]. For protein analysis, an equal volume of CM was mixed with Laemmli protein sample loading buffer and denatured at 95 °C. Fifteen microliter (15 μ l) of the denatured CM was loaded into a polyacrylamide-SDS criterion gel (10%) (Bio-Rad, CA, USA) and electrophoresis was run for 1.2 h. Proteins from the gel were electrophoretically transferred onto a PVDF membrane, which was blocked with 5% non-fat milk. After blocking, the membrane was probed with primary antibody (clone 22C11, Chemicon, MA, USA) against the N-terminus 66–81 residues of APP, and chemoluminescence signals were obtained. Protein band density was quantified by 'Image J' software (NIH, MA, USA). Afterwards, the same blot was stripped off by 'Re-probe

buffer' (Pierce, IL, USA). The stripped blot was re-blocked in 5% non-fat milk, probed with secondary antibody (goat anti-mouse) and exposed in an X-ray film to ensure that previous primary antibody (22C11) was completely removed. The blot was probed with monoclonal antibody clone 6E10 (Covance, NJ, USA), which detects 1–17 residues of the A β sequence within the APP molecule. The specific protein band signals were obtained and analyzed in the same way. Bradford protein assay was performed to measure total protein content of cell lysate (CL) samples, and an equal amount of CL protein was loaded in another 'Criterion' western blotting gel to detect total intracellular APP using 22C11 antibody. To normalize the APP signals, a specific strip of the blot was also probed with β -actin antibody (Sigma). Separately, A β (1–40) (N) ELISA assay was carried out with 50 μ l of each CM sample, as per the manufacturer's protocol (IBL, Takasaki, Japan). A β (1–42) levels were also measured independently using an IBL ELISA kit with 50 μ l of the CM samples. However, A β (1–42) levels were below the detection level in these samples. Similar independent experiments in duplicate ($n=2$) were performed at a different time. Analyses of Western blot and ELISA were carried out by combining two independent experiments performed in duplicates, i.e. ($n=2$) into one quadruplet ($n=4$) dataset. For this, data of each individual experiment were divided by the mean of the entire dataset of the same experiment. Statistical significance was determined by repeated measure ANOVA and independent t -test using SPSS 16.0 edition.

Memantine (10 μ M) was not toxic to the SK-N-SH cells and it produced a significant decrease in the levels of both sAPP and sAPP α at all time point studied (Fig. 1a and b). Significant decreases (approximately 50%) in levels of total sAPP and sAPP α in the CM of memantine treated cells were evident even at as early as 8 hour post treatment. These decreases were not due to an uneven transfer of proteins onto the membrane as assessed by Ponceau stain (Fig. 1a and b). Because of an increase in cell number and metabolic activity of cells, levels of sAPP and sAPP α in both vehicle and memantine treated cells increased gradually with the increase in duration of treatment. However, we quantified a non-significant slight increase in levels of total intracellular APP in the CL samples of memantine treated cells vs. vehicle treated cells after of

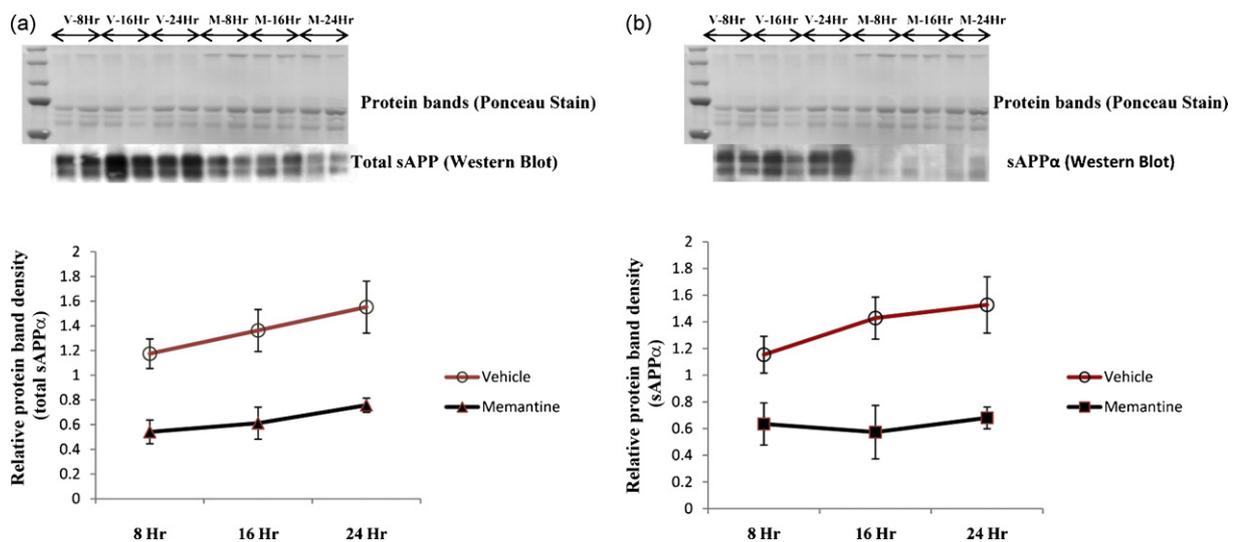


Fig. 1. Levels of sAPP and sAPP α by Western immunoblotting of conditioned media (CM) samples from the human neuroblastoma cells (SK-N-SH) treated with vehicle or memantine at different time points: (a) The Western blot strip showed the protein bands belonging to total sAPP in memantine and vehicle treated CM samples. Levels of secreted total APP showed a decrease in memantine treated cells vs. vehicle treated cells. A significant decrease in sAPP level was noted after 8 h of treatment, which continued throughout the time course of the study. A protein band picture (Ponceau stain) of the blot after transfer is also shown, which indicates uniformity of protein loading in the gel or transfer onto the membrane. Results are plotted from four independent ($n=4$) sets of experiments and a representative Western blot with two sets of experiments is shown. (b) Secreted APP α was measured from the same blot after stripping off signals of 22C11 antibody, as mentioned in (a). The Western blot picture shows a decrease in sAPP α levels in memantine treated samples vs. vehicle. This finding is similar to sAPP, which is shown in (a) ('V' represents 'vehicle' and 'M' represents memantine).

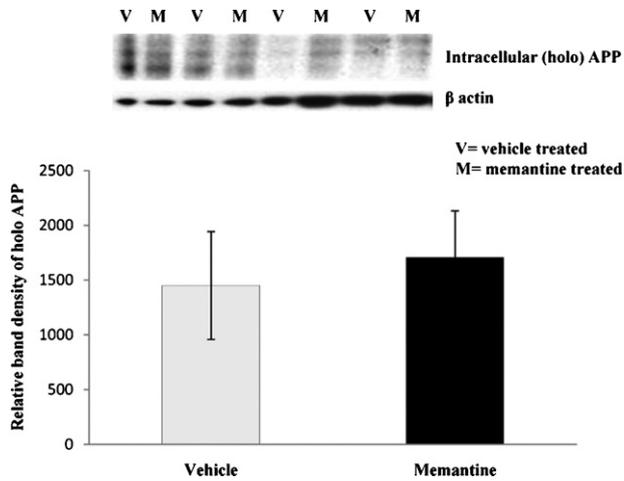


Fig. 2. Levels of total APP by Western immunoblotting of cell extracts from the human neuroblastoma cells treated with vehicle or memantine: Western blot data showed a slight non-significant ($p = 0.701$) increase in intracellular total APP (holo APP) levels in the lysate of memantine treated cells vs. vehicle. The relative density of each band was normalized with β -actin band, as indicated. Cell lysates from four experiments were analyzed.

24-h treatment period (Fig. 2). Interestingly, secreted $A\beta_{(1-40)}$ ELISA data showed a decrease in levels in the CM samples of memantine treated cells (Fig. 3). Results from the CTG assay revealed no change in cell viability between memantine and vehicle treated groups (Fig. 4a). Previously, we have reported a significant increase in cell viability of memantine treated embryonic primary cortical neurons [1]. However, such primary cortical neuronal cells were treated with memantine in nutritionally enriched medium. In our present experiment, memantine treatment was carried out in low serum medium, which restricts multiplication of cells. Under these conditions, there proved to be no significant change in toxicity in memantine treated cells, assessed by detecting LDH in the CM samples (Fig. 4b) suggesting that memantine was well tolerated in our experimental condition.

APP is a transmembrane glycoprotein that contains 695, 751 or 770 amino acid residues depending upon the splice variance [22]. Synthesis of APP together with its N-terminal glycosylation occurs within the endoplasmic reticulum (ER), immature APP then moves to the Golgi apparatus for maturation and, thereafter, it eventually is transported towards the cell membrane [10]. APP can be cleaved by α or β secretase enzyme activities to generate sAPP α and a C99 fragment, respectively, and the latter can be cleaved by presenilin dependent γ secretase to produce $A\beta$ peptide of varying amino acid

sequences. It is thought that cleavage of APP and subsequent generation of $A\beta$ peptide occurs in subcellular acidic pH of Golgi and endosomes [31] or at the cell surface [3]. Any alteration in the trafficking of the APP molecule may result in a deficient production of total sAPP, sAPP α and $A\beta$ peptide. In our study, we observed a greater than 50% decrease in the levels of both sAPP and sAPP α in each of the three time points studied within a 24-h time period. Western blot data from both vehicle and memantine-treated cells clearly revealed that the decrease in levels of sAPP and sAPP α in memantine treated cells started at or before the 8-h time period, and remained significantly lower during the rest of the treatment duration (for 24 h). Additionally we observed a slight increase in intracellular total APP in the memantine treatment group. Although this effect of memantine was not significant, in a separate experiment, we observed a significant increase in intracellular APP levels with 20 μ M dose of memantine (data not shown). These circumstances may indicate that more APP is available in the cells, which may act as a substrate for β followed by γ secretase cleavage. Beta (β) and γ secretase cleavage of the APP molecule would be reflected as an increase in $A\beta$ formation. Interestingly, we observed a significant decline in levels of the secreted N terminal fragment of $A\beta_{(1-40)}$ in the CM of memantine treated cells. Soluble oligomers of $A\beta$ peptide have been shown to trigger Ca^{++} influx through NMDA receptors, which ultimately could lead to excitotoxic damage of cultured hippocampal neuronal cells and, interestingly, this can be prevented by memantine [6]. Furthermore, 4 mM of Mg^{++} , which is a low affinity NMDA receptor antagonist, has been shown to decrease $A\beta$ burden in organotypic hippocampal cultures from APP/PS1 transgenic mice [11]. In this regard, a 'pleiotropic' property of memantine has recently emerged as it has been documented that memantine treatment reduced $A\beta$ plaques in APP/PS1 transgenic mice [28] and lowered the levels of $A\beta_{(1-40)}$ and secreted APP in human SK-N-SH cells [1]. In addition to neuronal cells, we have recently observed a decrease in the levels of total sAPP and $A\beta$ in rat glioma (C6) cell cultures (Ray and Lahiri, unpublished data). The exact mechanism mediating memantine's role in the reduction of APP and $A\beta$ remains to be fully delineated as our described studies have not measured the levels of sAPP α or total intracellular APP, and thus it would be interesting to know whether memantine directs APP processing towards a non-amyloidogenic pathway or alters the level of total APP message. In our present study, we utilized undifferentiated human SK-N-SH cells, which lack functional NMDA receptors and do not show noticeable calcium influx when treated with NMDA [25]. Hence, the effect of memantine on APP processing may involve a component that is non-NMDA receptor mediated and we consider the following additional possibilities:

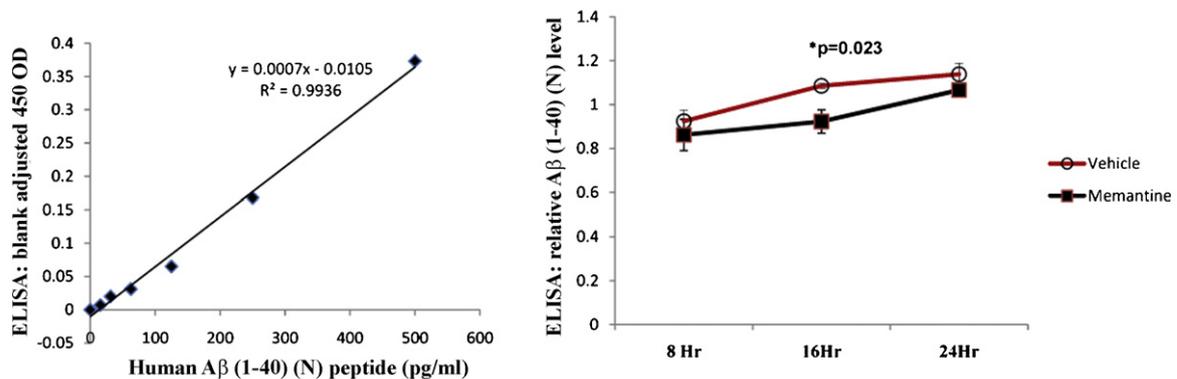


Fig. 3. ELISA determination of secreted $A\beta_{(1-40)}$ (N) peptide in conditioned media (CM) samples at different time points: levels of N-terminus fragment of secreted $A\beta_{(1-40)}$ were measured from the CM samples of memantine and vehicle treated SK-N-SH cells by ELISA as per the manufacturer's protocol. A standard curve performed with a known amount of N-terminus human $A\beta_{(1-40)}$ is also shown. The amount of detected $A\beta_{(1-40)}$ peptides in the CM range from 57 to 97 pg/ml. Results showed a decreasing trend of $A\beta_{(1-40)}$ in the CM of memantine treated cells. A statistically significant decrease was noticed at the 16-h time point ($P = 0.023$).

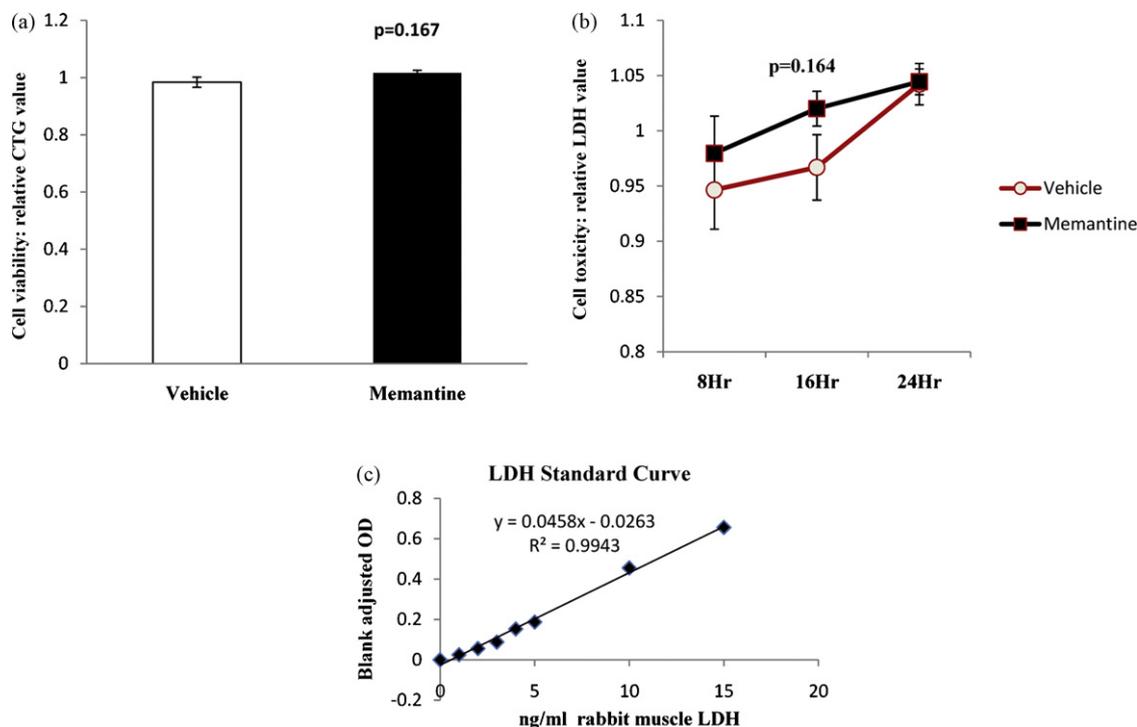


Fig. 4. Assay of viability and toxicity in the cells treated with vehicle or memantine for 24 h: (a) After memantine treatment, cells were lysed by adding 500 μ l of 'IP' buffer to obtain cell lysate. Equal volumes (30 μ l) of cell lysates were mixed with an equal volume of CTG assay buffer, and luminescence signals were obtained in a luminometer. Data are plotted as relative luminescence units combining both experiments. CTG or cell viability data did not show any significant change between the memantine and vehicle treated groups. (b) CM samples of memantine and vehicle treated cells were tested for membrane damage or toxicity using the LDH method. In our assay, LDH activity ranged from 0.8 to 1.08 ng/ml. Data plotted in the figure are relative LDH values combining two sets of identical experiments (relative value). We did not observe any significant toxic effect of memantine treatment vs. the vehicle. (c) LDH standard curve with known amount of LDH from rabbit muscle shows the linearity of the assay.

Following *N*- and *O*-glycosylation, nascent APP travels towards the cell surface followed by internalization into the endosomes where it can become the substrate for BACE-1 cleavage [30]. In contrast, APP is processed by α -secretase at the cell surface. Since we have found a decrease in levels of total sAPP, sAPP α and A β and a concomitant slight increase in levels of total intracellular APP, we speculate that memantine might play a role in the trafficking of APP molecules by either keeping immature APP in endoplasmic reticulum (ER) for a longer time or increasing its trafficking towards lysosomes. Lysosomal accumulation of memantine has already been proposed, which could provide an explanation to account for the reduced APP and A β secretion in memantine treated cells [1]. In this context, cholesterol depleting agents have been shown to reduce A β production by interfering with intracellular APP trafficking [9,10]. Similarly, the ChEI tacrine has been shown to effect the trafficking of APP to a lysosomal compartment [19] and has therefore been suggested to have a lysosomotropic property [15]. Memantine has also been shown to reduce BACE activity in cultured human neuroblastoma cells [13]. Taken together, our results suggest that memantine decreases levels of secreted APP, sAPP α and A β _(1–40) and may retain intracellular APP in human SK-N-SH cells. These results warrant further studies on the mechanism of action of memantine on the APP processing pathway.

Acknowledgements

We thank B. Maloney, J. Bailey and J. Long, and grants support from the Alzheimer's Associations (Zenith Award) and the National Institutes of Health (AG18379 and AG18884) to DKL, as well as by the Intramural Research Program, National Institute on Aging to NHG.

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