The enhancement effect of beta-boswellic acid on hippocampal neurites outgrowth and branching (an in vitro study)

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Abstract Increasing evidences implicate impairment of axonal integrity in mechanisms underlying neurodegenerative disorders. Beta-boswellic acid (BBA) is the major component of Boswellia serrata gum. This resin has long been used in Ayurveda (India’s traditional medicine) to prevent amnesia. In this study, the effect of BBA was examined on neurites outgrowth and branching as well as on polymerization dynamics of tubulin. The morphometric parameters (axonal length and neuritis branching) were examined microscopically after treating the hippocampal cells with BBA. Also the assembly process of tubulin was assessed using UV/V is spectrophotometer through following of absorbance at 350 nm. The results revealed that BBA could significantly enhance neurite outgrowth, branching, and tubulin polymerization dynamics. The obtained results suggest that enhancing effect of BBA on microtubule polymerization kinetics might be the origin of increasing axonal outgrowth and branching.

Keywords Axonal outgrowth and branching · Beta-boswellic acid · Microtubule polymerization dynamics

Introduction

Development of neuronal circuits and networks is supported by dynamic instability of microtubule proteins (MTPs) [1]. This property plays a crucial role in microtubule biological functions such as mitosis, meiosis, cell motility, axonal vesicles transport, and axonal extension [2]. Disruption of microtubule structure impairs the axonal transport, synaptic loss, and neuronal cell death which are known to be the leading causes for neurodegenerative disorders such as Alzheimer’s disease (AD) [3]. AD, a chronic and progressive neurodegenerative state, is the leading cause of dementia in the elderly [4]. The presence of neurofibrillary tangles (NFTs), senile plaque (SPs), neuronal death, synaptic loss, and brain atrophy in specific brain areas, has been shown to be hallmarks of AD [5]. Although NFTs are insufficient to cause cell death, they have a principal role in dementia severity and amyloid plaques formation [6, 7]. Neurofibrillary alterations and neuronal death are not observed in transgenic animals with amyloid precursor protein (APP) mutations, but tau protein has been shown to be essential in AD [8]. Moreover, impairment of axonal microtubule causes memory loss [9]. Therefore, the key factor which induces memory loss and impairment in AD patients could be neurite degeneration through MTP destabilization [10].

The gum resin of plants Boswellia species from Burseraceae family, so-called Olibanum, has long been used for prevention of amnesia and enhancement of memory power in India’s traditional and natural system of medicine (Ayurveda) as well as Oriental Medicine. Ayurveda, which has been practiced for more than 5,000 years, provides an integrated approach for preventing and treating illness through lifestyle interventions and natural therapies [11, 12]. Chewing the Boswellia gum by pregnant women,
adults, and elderly was highly recommended by the Persian physician, Avicenna in The Canon of Medicine (The Law of Medicine). Also the gum resin of *Boswellia* has been demonstrated to improve remedy of rheumatoid arthritis and inflammation [13]. Ethanolic extract of gum resin of *Boswellia serrata* has been recognized to inhibit formation of Leukotriene B4 which is one of the important mediators in inflammatory reactions [14].

Gum resin consists of different terpenoid compounds, and beta-boswellic acid (BBA) has been reported to be the major component [15]. Because of the essential role of microtubule in axonal guidance, neurodegenerative disorders, and neural plasticity and in addition the importance of *Boswellia* resin as seen in Ayurvedic and Oriental Medicine in prevention and remedy of memory-associated diseases, in the current research we planned to identify the effect of major component of this resin (BBA) on hippocampal cell type growth and polymerization dynamics of MTP.

**Materials and methods**

**Reagents**

Ethylenebis (oxyethylenenitrilo) tetraacetic acid (EGTA), guanosine 5′ triphosphate type II-S (GTP), Adenosine 5′ triphosphate (ATP), phenylmethylsulphonyl fluoride (PMSF), glycerol, dimethyl sulfoxide (DMSO), MgSO₄, and colchicine were purchased from Sigma (Deisenhofen, Germany). Piperazine-1,4-bis(2-ethanesulfonic acid) (PIPES) was purchased from Merck (Darmstadt, Germany). Phosphocellulose type P11 was prepared from Whatman. BBA was a generous gift from Prof. Thomas Simmet (Department of Pharmacology of Natural Products and Clinical Pharmacology, University of Ulm, 89081 Ulm, Germany). It was dissolved in DMSO as a vehicle to 20 mM final concentration. MgSO₄ solution (1 M) was added to each GTP and ATP stock solutions (100 mM) as a ratio of 1:10 (v/v). Deionized and nanopure water was used in all experiments.

**Animals**

Adult female pregnant Sprague-Dawley rats (120–200 days old) were maintained on a 10–14 h light–dark cycle with food and water available ad libitum. Adult females were caged with males overnight and checked for the presence of a vaginal plug on the following day. The day on which the plug was found was designated as the first day of pregnancy [16, 17]. Animals were killed by rapid decapitation on the 17th day of pregnancy to obtain hippocampal sample from the fetuses.

**Hippocampal cell culture**

Primary cells of hippocampal tissue were cultivated at low density as previously described [17]. Briefly, the hippocampus was dissected and pooled together in a sterile Petri-dish containing Mg²⁺/Ca²⁺-free Tyrode’s buffer (137 mM NaCl, 4 mM KCl, 0.36 mM NaH₂PO₄, 0.18 mM KH₂PO₄, 12 mM NaHCO₃, 11 mM glucose). The hippocampal tissue was then diced into 1–3 mm cubes and washed twice with 20 volumes of Mg²⁺/Ca²⁺-free Tyrode’s buffer. Cells were pelleted down by centrifugation at 325×g for 5 min. The pellet was incubated in 1 ml of trypsin/EDTA at 37°C for 15 min for enzyme digestion. The reaction was stopped by addition of 2 volumes Dulbecco’s modified eagle medium (DMEM) containing 10% FBS. Cells were then mechanically triturated using three different sizes of sterile Pasteur pipette attached to a Pi-pump. Cell suspensions in DMEM containing 10% FBS and 1% Pen-strep were seeded onto cover slips. After 3–4 h, cover slips were transferred into 24-well multidishes. The cover slips were placed with the neurons facing downward. Cultures were maintained at 37°C in a humidified atmosphere containing 95% air and 5% CO₂. The day after planting, DMEM was replaced with serum-free media (DMEM, 1% Penstrep) containing 10, 20, 30 nM BBA, and 20 nM colchicine as negative control (12 well/dose). To examine the effect of dose, on day 8, cell morphology was evaluated [18, 19]. Cells in five wells were chosen randomly on day 8, and 10 fine photos were captured from each well (50 images/dose) with phase-contrast microscope (Zeiss Axiovert 25, Jena, Germany). 20 out of 50 images were selected randomly for further analysis.

**Image analysis and statistics**

Sigma Scan software 3.0 (Jandel Scientific; SigmaScan Pro) was used for manual tracing analysis. Morphometric parameters (axonal length and neurites branching) were analyzed for statistical significance using a two-tailed Student’s *t* test [20]. The mean values of the morphometric changes were considered significant at *p* < 0.05.

**Tubulin purification**

Microtubule protein was prepared from two sheep brains, after homogenization in the appropriate buffer (100 mM PIPES pH 6.9, 1 mM EGTA, 1 mM MgSO₄, 1 mM PMSF and 1 mM MgATP) followed by two cycles of temperature-dependent assembly and disassembly induced by addition of 1 mM MgGTP, as previously...
described [21]. PEMG (100 mM PIPES pH 6.9, 2 mM MgSO₄, 1 mM EGTA, and 3.4 M glycerol) was used as polymerization buffer. To obtain purified and MAP-free tubulin (PC-tubulin), the crude tubulin sample was applied to a phosphocellulose column, and eluted tubulin fractions were promptly frozen in the liquid nitrogen and stored at −70°C for further experiments within 2 weeks. The resulting tubulin was essentially pure (more than 97%) as determined by Coomassie Blue staining of sodium dodecyl sulfate polyacrylamide (SDS-PAGE) gel 10% [22]. The protein concentration was determined by using the Bradford reagent with bovine serum albumin (BSA) as the protein standard [23].

Turbidimetric analysis of MTP polymerization

A Cary-100 Bio-Varian (Australia) UV/V is spectrophotometer was programmed to follow and monitor assembly reactions by recording of absorbance at 350 nm every 20 s. To study tubulin assembly, stored MTP aliquots were thawed and clarified by centrifugation (30,000×g, 15 min, and 4°C) to remove tubulin aggregates and diluted to final concentration of 1.5 mg/ml in PEMG buffer [24].

Results

Effect of BBA on axonal outgrowth and branching

There are wide agreements that net addition of new microtubule polymer to the axon is necessary for its growth [25]. Consequently, in the current work in order to investigate effect of BBA on both axonal outgrowth and branching, the hippocampal cells were treated with three different doses of this compound (10, 20 and 30 nM) for a time period of 8 days, according to the method described in “Materials and methods”. The results as shown in Figs. 1a–d, 2 and also in Table 1 indicate significant effects of BBA on both axonal outgrowth and branching. As seen in Fig. 1d, when the highest concentration of BBA (30 nM) was applied, a marked effect on both axonal outgrowth and branching was seen.

MTP assembly in the presence of BBA

The ability of microtubule to assemble and disassemble is critical for axonal outgrowth, branching, and guidance which must be finely regulated [26]. In this study as shown in Fig. 3, dynamics of MTP assembly was measured

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**Fig. 1** Effect of BBA on axonal outgrowth and branching. The embryonic hippocampal cells were treated with 20 nM colchicines (a), 10 nM BBA (b), 20 nM BBA (c), and 30 nM BBA (d) on day 8. Representative of 20 images for each treatment are shown. Bar 20 μm
turbidimetrically through following of absorbance at 350 nm. The concentration of PC-tubulin (1.5 mg/ml) was higher than its critical concentration, and BBA (0.15 and 0.30 mM) was incubated with unpolymerized tubulin protein on ice for 30 min before polymerization reaction initiated. Figure 3 shows following of the MTP assembly process for 23 min. The slope in the linear part of MTP polymerization curves has been suggested to be directly correlated with the rate of MTP polymerization [27]. As seen in Fig. 3, slopes of polymerization curves which calculated from their initial (linear) parts were significantly increased from 0.024 in the control experiment to 0.030 and 0.034 in the presence of 0.15 and 0.30 mM of BBA, respectively. This finding suggests an increase in the polymerization rate of MTP protein in the presence of BBA. On the other hand, time for achieving plateau (steady state) and also the maximum turbidity were 11 min and 0.14, respectively in the control experiment, whereas in the presence of 0.15 and 0.30 mM of BBA, achieving plateau was not seen as the assembly kinetics followed after 23 min.

It has been already reported that MTP length distribution is mutually related to maximum amount of turbidity which is also a reflection of the polymer mass or microtubule length [28]. As seen in Fig. 3, the maximum turbidity in the presence of 0.30 mM BBA is approximately two times higher than that was observed at the control experiment, showing a substantial increase of the average polymer mass or microtubule length induced after treatment with 0.30 mM BBA.

In a parallel experiment, as polymerization was proceeding, concentration of inactive tubulin fraction was measured every 3 min. The results of this experiment (Fig. 4) show that free tubulin concentration was decreased

<table>
<thead>
<tr>
<th>Samples</th>
<th>Length (pixel)</th>
<th>Branching (mean value number)</th>
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<tbody>
<tr>
<td>Control</td>
<td>311.15 ± 60.27</td>
<td>4.1 ± 1.19</td>
</tr>
<tr>
<td>20 nM Colchicine</td>
<td>94.64 ± 20.09</td>
<td>1.8 ± 0.78</td>
</tr>
<tr>
<td>10 nM BBA</td>
<td>492.57 ± 82.10</td>
<td>7.7 ± 3.23</td>
</tr>
<tr>
<td>20 nM BBA</td>
<td>653.19 ± 54.79</td>
<td>9.8 ± 4.10</td>
</tr>
<tr>
<td>30 nM BBA</td>
<td>802.56 ± 210.24</td>
<td>14.1 ± 5.78</td>
</tr>
</tbody>
</table>

Fig. 2 Effect of BBA on morphology of embryonic hippocampal cells. Quantitative analysis of the effect of different concentration of BBA on morphology of embryonic hippocampal cell type. The length of the axons and other neurites (a) and the number of branch points of axons and other neurites (b) in presence of colchicine and BBA, \( p < 0.05 \) compared to corresponding measurements in control cells.
during tubulin polymerization and confirmed that change in 
the turbidity was indeed the result of tubulin assembly and 
not protein aggregation. Consequently, changes in absor-
bance at 350 nm must be solely attributed to MTP poly-
merization. After considering all data presented herein, 
since BBA increases polymerization assembly, it can be 
concluded that BBA enhances the rate of net assembly of 
MTP protein which possibly leads to increased tubulin 
polymer stability.

Discussion

In the polymerization process of tubulin protein, the fre-
quency of switching from growing to the shortening is 
called catastrophe, whereas a transition from shortening to 
growing is named rescue [2]. Since BBA as shown in the 
current study could enhance the assembly of the microtu-
bule polymer, it is suggested that BBA could also increase 
either the rescue frequency and/or decrease the catastro-
phe.

As shown in the Fig. 3, BBA significantly increases both 
MTP net assembly kinetics and elevates the maximum 
turbidity in the polymerization curves, suggesting an 
increase of the polymer mass in the presence of BBA. This 
finding recommends greater stability in the presence of 
BBA of the cylindrical tubulin polymer which subse-
quently could affect the polymer length.

The crucial role of MTPs in establishing and maintain-
ing axonal structure has been documented before [29, 30]. 
As reported already microtubule polymerization or extru-
sion may by itself be able to drive axonal growth and pathfinding [31]. Since BBA increases both microtubule 
length and axonal outgrowth and branching, the results 
obtained in this study provide further supporting evidences 
for the idea suggesting a critical function of microtubule in 
the axonal growth. It has been reported before that an 
increase in stability of microtubule led to elevation of the 
polymer length and subsequently resulted in the increase of 
the axonal length [26]. On the other hand, axonal stability 
could be a reflection of stability of microtubule which may 
consequently prevent axonal degeneration. As reported 
previously axonal degeneration is in concomitance with 
microtubule disruption which is hallmark for neurodegen-
erative diseases and memory loss [9]. The significant 
increase of the microtubule polymerization dynamics and 
mass in the presence of BBA which consequently may 
only enhance the tubulin polymer stability might be at the origin 
of enhancement of the axonal growth and branching as 
observed in this study (Fig. 2a, b). Thus, the results of this 
in vitro study may suggest a possible remedial role for 
BBA as a useful reagent against neurodegenerative disor-
ders and the memory loss. Also increasing of axonal out-
growth and branching after treatment of the embryonic 
hippocampal cells with BBA may confirm the aforemen-
tonioned suggestion. Furthermore, in the Oriental Medicine 
the gum resin of Olibanum with BBA as the major com-
ponent has been recommended to prevent amnesia. Since 
this in vitro study revealed significant enhancing effect of
BBA on both neurite outgrowth and branching, further studies may clarify roles of BBA in the neural complexity and subsequently in the improving of the memory power.

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References