The Effects of Fasudil on Inflammation Induced by Amyloid Beta Peptide in an Astrocyte Cell Line

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ABSTRACT

Objective: Alzheimer's disease is a progressive neurodegenerative disorder with characteristic neuropathological changes. Neuropathological hallmarks of Alzheimer's disease include amyloid plaques and neurofibrillary tangles. The inflammatory process has a fundamental role in the pathogenesis of Alzheimer's disease. Cytokines play a key role in inflammatory and anti-inflammatory processes in Alzheimer's disease.

Rho-kinase is overactivated in many CNS disorders and its inhibition could be a potential therapeutic target for inflammatory and demyelinating diseases. Fasudil is a Rho-kinase inhibitor and has neuroprotective effects.

Methods: We aimed to investigate whether fasudil treatment represents a pharmacological approach in Alzheimer's disease by examining its effects in amyloid beta-induced inflammation in a murine astrocyte cell line and to contribute to existing literature.

Astrocytes were incubated with 5 μM amyloid beta for 24 hours. Another group of astrocytes was treated with Fasudil, a Rho kinase inhibitor, at a dose of 2.5 μM in addition to amyloid beta. cDNA synthesis was performed on RNA samples isolated from the cells. Gene expression analysis was conducted using real-time PCR methodology.

Results: Amyloid beta increased Tumor necrosis factor (TNF-α), IL-1β, IL-6, IL-10, IL-12, Cas-3, Cas-8, Bcl2-associated X protein (Bax) and B-cell CLL/lymphoma 2 (Bcl-2) mRNA expression levels 2 to 27-fold compared to control group. Fasudil treatment significantly reduced the increase in amyloid beta-induced inflammation and some apoptotic genes studied. (p<0.001).

Conclusion: Inhibition of Rho kinase by fasudil may be a potential treatment target owing to its protective effect against amyloid beta-mediated inflammation. However, further studies are needed to corroborate our preliminary findings.

Keywords
Alzheimer's disease, Amyloid beta, Fasudil, Cytokines.

Introduction
Alzheimer's disease (AD) is a neurodegenerative disease associated with loss of synapses an neurons in certain areas of the central nervous system (CNS) and it is characterized by progressive cognitive dysfunction, behavioral disturbances and impaired self-care abilities [1,2]. Each year, 4.6 million of new AD cases are estimated to occur globally [3]. The likelihood to develop AD increases in an age-dependent, logarithmic manner [4]. Epidemiological studies reported the multifactorial nature of Alzheimer's disease. Age-related neuronal disorders play an essential role in the development of AD. Additionally, head trauma, viral infections and metabolic lesions are associated with increased risk of AD [5]. Histopathologically, senile amyloid plaques (SP), formation of neurofibrillary tangles (NFT), loss of synapses and neurons and a marked brain atrophy are common in
AD (4). NFTs cause cell death by impairing cytoskeletal integrity and axonal transport dynamics [6].

Another neuropathological change in the AD brain is amyloid plaques, of which amyloid beta (Aβ) is the main component. Aβ is a protein of 40-42 amino acids that is crucially involved in Alzheimer’s disease as the main component of amyloid plaques. Through proteolytic processing, Aβ is generated from the amyloid precursor protein (APP) of undetermined function which is a large transmembrane glycoprotein encoded by chromosome 19 [6,7].

An acute phase response occurs in AD due to degeneration of the brain. This stimulates a rapid rise in the proteins called “cytokines” which activate the immune defense mechanisms of the body [8,9]. Microglial cells are the primary phagocytic cells in the brain parenchyma. When activated in response to brain pathology, they function like macrophages and assume multiple roles including phagocytosis, antigen presentation and release of several inflammatory and neurotoxic factors. In vitro experiments have shown that inflammatory cytokines released by microglial cells lead to neuronal damage [10].

Rho kinase (ROCK) is a small G protein involved in many intracellular signaling pathways and has two effectors, namely ROCKα/ROCK II and ROCKβ/ROCK I [11]. ROCK plays a role in several physiological events including blood pressure homeostasis, vascular smooth muscle contraction, cell migration, adhesion and proliferation, apoptosis and inflammatory response. Apart from its typical involvement in the regulation of actin cytoskeleton, ROCK has a critical role in cell migration, chemotaxis, adhesion, generation of reactive oxygen species (ROS) and apoptosis [12]. Fasudil is an agent that inhibits ROCK [13]. Recently, Fasudil was reported to protect the brain tissue against ischemic injury [14]. Initially identified as an intracellular calcium antagonist, Fasudil has been used in Japan for a while for the treatment of cerebral vasospasms after subarachnoid hemorrhage [15]. The mechanisms involved in Fasudil’s actions against cerebrovascular occlusion are thought to include increased cerebral blood flow and reduced inflammatory response [14,16]. Decreased inflammatory response induced by ROCK blockage occurs via inhibition of neutrophil migration [16].

In this study, we aimed to investigate whether fasudil treatment represents a pharmacological approach in Alzheimer’s disease by examining its effects in amyloid beta- induced inflammation in a murine astrocyte cell line.

Materials

Cell Culture

For this study, C8-D1A (a murine astrocyte cell line) was used to construct a neurotoxicity model induced by Aβ peptide. C8-D1A cell line was obtained from the American Type Culture Collection (ATCC), underwent repeated passages at our laboratory and stock solutions were stored in liquid nitrogen.

The cell count of a specific cell population was determined quantitatively using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) by spectrophotometric method.

The appropriate dose was chosen as 5 µM Aβ based on statistical data and literature review. MTT was conducted at different doses for Fasudil (2.5 µM-80 µM) and the appropriate dose was determined as 2.5 µM based on statistical analyses.

In our study, ethics committee approval wasn’t obtained because human or animal material was not used.

RNA Isolation

25 cm² flasks were inoculated with 2x106 cells. One day later, three groups of cells were treated with either Aβ, fasudil or Aβ plus fasudil and a control group was left untreated. After 24-hour incubation flasks were transferred to the safety cabinet and all of the media contained in the flasks was discarded. Total RNA extracted using Trizol RNA Isolation Protocol.

Real-Time Polymerase Chain Reaction (RT-PCR) Analyses

Transcription levels of TNF-α, IL-1β, IL-6, IL-10, IL-12, BCL2, BAX, Cas-3 and Cas-8 were determined using RT-PCR method.

1 µg RNA was obtained from each sample to be used as a template in the PCR reaction and complementary DNA (cDNA) synthesis was performed with reverse transcriptase (RT). The instructions described in the complementary DNA kit were followed for this procedure.

Following cDNA recovery, 1 µl of cDNA from each sample was incubated with 6 µl SYBR Green master mix (Power SYBR Green PCR master mix 5 ml), forward and reverse primers (0.3 µl each; using primer sequence designs shown in Table 1) and 4.4 µl RNase-free water using a suitable protocol design and temperatures and times shown in Table 2. DNA amplification was performed with PCR methodology using Applied Biosystems Step One Plus real-time PCR systems. After amplification, cycle threshold (Ct) values for the amplification curves were compared and relative changes in mRNA transcription levels of the genes of interest were calculated using the 2-DDCT method.

For this calculation the following formula was used:

\[ \text{DDCt} = \frac{(\text{Ct} - \text{Ct} - \beta\text{-actin})_{\text{gene of interest}}} {\text{sample group}} - \frac{(\text{Ct} - \text{Ct} - \beta\text{-actin})_{\text{gene of interest}}} {\text{control group}} \]

To find out the fold gene expression, mRNA transcription level was expressed as upregulated or downregulated by including 2 to the power of negative delta-delta CT to the resulting values for each gene. β-actin gene (housekeeping gene) was included in the reaction as a correction factor for each RNA sample. In the Real Time-PCR system, SYBR Green I dye preferentially binds to double-stranded DNA fragments and emits fluorescent light; the emitted light is captured by the laser detector of the system and the computer software interface interprets the detector signal. The intensity of the fluorescent light is directly proportional to the amount of the PCR product. Differences in mRNA transcription...
between study groups are shown in the following figures.

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Forward primer</th>
<th>Reverse primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-Actin (Beta aktin)</td>
<td>Housekeeping gene</td>
<td></td>
</tr>
<tr>
<td>TNF-α (Tumor Necrosis Factor- alpha)</td>
<td>5’-AGCCGATGGGTGTTACCTTGTC-3’</td>
<td>5’-TGGAGATAGCATACTCGCT-3’</td>
</tr>
<tr>
<td>IL-β (Interleukin 1 beta)</td>
<td>5’-TTTGAGCTGGAGAGGCTG-3’</td>
<td>5’-AACGTCACACACGACCCAGT-3’</td>
</tr>
<tr>
<td>IL-6 (Interleukin 6)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-1β (Interleukin 1 beta)</td>
<td>5’-ATCCAGTTGCCTCTTGGAGACTGA-3’</td>
<td>5’-TAAGGCTCCGATCTGGTGAAGTG-3’</td>
</tr>
<tr>
<td>IL-1β (Interleukin 1 beta)</td>
<td>5’-CTGGGAGATGCCACTTTG-3’</td>
<td>5’-CATGGGATCTGGTTCTTTGC-3’</td>
</tr>
<tr>
<td>BAX (BCL2-associated X protein)</td>
<td>5’-ATGTTGTTGAGAGGCTG-3’</td>
<td>5’-ACAGTCTCCAAGGGCATCC-3’</td>
</tr>
<tr>
<td>BCL2 (B-cell CLL/lymphoma 2)</td>
<td>5’-CTGGGAGATGCCACTTTG-3’</td>
<td>5’-CATGGGATCTGGTTCTTTGC-3’</td>
</tr>
<tr>
<td>(CAS-3) Caspase-3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(CAS-8) Caspase-8</td>
<td></td>
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</tr>
<tr>
<td>IL-12 (Interleukin-12)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-10 (Interleukin 10)</td>
<td></td>
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</tbody>
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Table 1: The list of primer sequences used for gene expression analyses using RT-PCR.

Applied Biosystems StepOnePlus real-time PCR systems were used for the study; Table 2 shows the details of the PCR protocol used.

<table>
<thead>
<tr>
<th>Temperature</th>
<th>Time</th>
<th>Step</th>
<th>Number of Cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>95°C</td>
<td>5 min</td>
<td>Initial denaturation</td>
<td>Only 1</td>
</tr>
<tr>
<td>95°C</td>
<td>15 sec</td>
<td>Denaturation</td>
<td>40 cycles</td>
</tr>
<tr>
<td>60°C</td>
<td>40 sec</td>
<td>Annealing</td>
<td></td>
</tr>
<tr>
<td>70°C</td>
<td>45 sec</td>
<td>Extension</td>
<td></td>
</tr>
</tbody>
</table>

Table 2: PCR Protocol of Applied Biosystems StepOnePlus Real-Time PCR Systems.

Results

Varying concentrations (2.5-80 μM) of Aβ were added into 96-well cell culture plates containing the astrocyte cell line (C8-D1A cells) and the plates were incubated for 24 hours. At the end of 24 hours, viability checks were conducted using the MTT method. A marked reduction in viable cell count was observed in the group exposed to 5 μM Aβ in comparison to control group.

Throughout the study, 5 μM concentration of Aβ was used to induce stress in the cells. Fasudil was added to astrocytes in 96-well cell culture plates at varying concentrations (2.5-80 μM) and cell viability was evaluated using MTT method to determine the effective concentration. The cell viability of control was set to 100% and the following percent viability values were observed: approximately 36,20 for Aβ 5 μM, 51,89 for Fasudil 2.5 μM and 40,86 for Aβ+Fasudil. TNF-α mRNA expression was upregulated by 221% in the group exposed to amyloid beta versus control group but fasudil treatment reduced these levels by %28 (Figure 1).

IL-1β mRNA expression was 177% higher in the group treated with amyloid beta versus control group but amyloid beta plus fasudil treatment reduced these levels by 51% (Figure 2).

mRNA expression of IL-12 gene was upregulated by 98% in the group exposed to amyloid beta compared to control group but this increase was suppressed by 70% when fasudil was added to amyloid beta (Figure 3).

IL-10 mRNA expression was 221% higher in the group treated with amyloid beta versus control group and fasudil treatment suppressed this increase by 37% (Figure 4).
IL-6 mRNA expression was upregulated by 193% in the group exposed to amyloid beta versus control group but fasudil suppressed this increase by 27% when given together with amyloid beta (Figure 5).

![Figure 5. Cas-3 mRNA expression levels (at 24 hours)](image)

Cas-3 mRNA expression increased by 278% in the group exposed to amyloid beta versus control group but fasudil suppressed this increase by 174% (Figure 6).

![Figure 6. Cas-3 mRNA expression levels (at 24 hours)](image)

Cas-8 mRNA expression was 348% higher in the group treated with amyloid beta compared to control group and fasudil suppressed 110% of the increase induced by amyloid beta (Figure 7).

![Figure 7. Cas-8 mRNA expression levels (at 24 hours)](image)

Bel-2 mRNA expression level was 282% higher in the group exposed to amyloid beta; however, fasudil suppressed this increase by 53% when administered with amyloid beta (Figure 8).

![Figure 8. Bel-2 mRNA expression levels (at 24 hours)](image)
mRNA expression of the Bax gene was upregulated by 200% in the group treated with amyloid beta and fasudil suppressed this increase by 100% when added to amyloid beta (Figure 9).

![Figure 9. Bax mRNA expression levels (at 24 hours)](image)

**Discussion**

Recent studies have shown that astrocytes are activated by Aβ and this in turn, leads to release of inflammatory factors that impair synaptic and neuronal health in AD models [17-19]. Astrocytes are essential for Aβ-induced tau phosphorylation and elicit the expression of proinflammatory cytokines including TNF-alpha, IL-1β and IL-6 [18].

Experimental evidence demonstrates that neuroinflammation plays an active role in the development and progression of AD [20,21]. Aβ is known to activate astrocytes and microglia via induction of inflammatory signal transmission [17,18]. Both microglia and astrocytes release numerous pro- and anti-inflammatory cytokines under different conditions. In this study, the effectiveness of Fasudil in preventing cell death was investigated in the setting of Aβ-induced inflammation. For this purpose, Alzheimer’s disease was induced in a murine cell line and cell viability tests were performed. While extensive cell death was observed in the astrocyte group treated with 5 μM Aβ after 24-hour incubation compared to control group, cell loss was significantly reduced in the astrocyte group exposed to 5 μM Aβ plus 2.5 μM Fasudil. Satoh et al. explored the effects of delayed administration of Fasudil on ischemia-induced cell death in the hippocampal CA1 region of gerbils and demonstrated protective effect of Fasudil (10 mg/kg) against delayed ischemic neuronal damage induced by edavarone (3, 10 mg/kg) [20].
Proinflammatory cytokines induced by Aβ may produce pro-apoptotic and synaptotoxic effects which are directly harmful to neurons [21]. Moreover, there is evidence that inflammation contributes to formation of Aβ aggregates and senile plaques and plays a key role in the pathogenesis of AD [22]. Inhibition of proinflammatory cytokine production stimulated by Aβ represents a plausible strategy for developing therapies for prevention and treatment of AD. Yung Son et al. [23] found that when administered into the hippocampus of rats, fasudil reduced elevated IL-1β and TNF-α production induced by Aβ via ROCK inhibition.

IL-6 immunoreactivity in AD senile plaques has been previously reported [24]. More recently, higher concentrations of IL-6 were detected in AD brains using biochemical methodology [25]. Additionally, IL-6 was demonstrated to cause neurodegeneration in transgenic mice in the absence of amyloid pathology [26].

Apoptosis is characterized by activation of caspase-dependent mitochondrial dysfunction which mediates proteolytic degradation of cytoplasmic and nuclear proteins, nuclear condensation, DNA degradation and eventually cell death [27]. The core effectors of the apoptotic process encompass proteases known as caspsases (cysteine-dependent aspartate-directed proteases) [28,29]. Caspsases are latent precursors in most nucleated animal cells which initiate programmed death and when activated, they disassemble the internal infrastructure of the cells and activate factors that are harmful to the cells. In a murine model of familial Alzheimer’s disease, D’Amelio et al. [30] analyzed caspase-3 activity in the hippocampal dentritic spines of transgenic mice carrying a mutant allele (Tg2576-APPswe) and found increased caspase-3 activity at 3 months before occurrence of detectable amyloid plaque deposits at these synapses [31]. Bcl-2 proteins reside on the outer mitochondrial membrane. They have the ability to protect cells from a variety of apoptotic stimuli including oxidative stress. Nuclei exhibiting DNA damage within neurons in the AD brain were reported to be associated with upregulation of Bcl-2 expression [32]. Bcl2-associated X protein (Bax) is a member of the Bcl-2 family and expressed in the neurons of the peripheral nervous system [33]. Overexpression of Bax protein is known to induce apoptotic cell death [34]. In the present study, the expression levels of proinflammatory cytokines IL-1β, TNF-α, IL-12, IL-6, and IL-10 and apoptotic genes cas-3, cas-8, Bcl-2 and Bax were analyzed in astrocytes exposed to Aβ for 24 hours. As a result, Aβ was found to significantly increase the expression of these cytokines.

**Conclusion**

These findings and reports from previous studies suggest that Fasudil might be used as a therapeutic agent in Alzheimer’s disease. In this study, the anti-inflammatory effect of Fasudil, a Rho-kinase inhibitor, was investigated in an Aβ-induced inflammation model in murine C8-D1A astrocyte cell line. We demonstrated that Aβ caused extensive cell death by potently inducing inflammation, whereas Fasudil effectively suppressed Aβ-induced cell loss. These findings suggest that Fasudil may be used as a therapeutic agent for AD owing to its suppressive effect on inflammation elicited by amyloid beta. We hope that our results will, to some extent, guide future in vitro research studies examining whether Fasudil can be used as a pharmacological approach for the treatment of Alzheimer’s disease and fill a gap in the relevant literature.

**References**


