RESEARCH ARTICLE

Assessment of cell viability in an astrocyte cell line exposed to amyloid beta peptide and Fasudil

Burçin Nilay Yener[1*], Necla Benlier[2], Hülya Çicek[1]

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Background: Amyloid beta (Aβ) is a protein of 40-42 aminoacids that are crucially involved in Alzheimer's disease as the main component of amyloid plaques. Rho-kinase is believed to be involved in the regulation of stress fiber formation, smooth muscle contraction, cell migration and proliferation. Rho-kinase inhibition results in reduced inflammatory response. Fasudil is a ROCK inhibitor. In the present study, we aimed to evaluate cell viability in an astrocyte cell line treated with amyloid beta peptide and fasudil.
Methods and results: C8-D1A (a murine astrocyte cell line) was used to construct a neurotoxicity model induced by Aβ peptide. Astrocyte cells were divided into 2 groups including control and amyloid beta groups and incubated with 5 μM amyloid beta for 24 hours. A separate group was treated with 2.5 μM dose of Fasudil, a Rho-kinase inhibitor, and photographs of the resulting cell samples were taken with 40X magnification objective of the inverted microscope.
A marked increase in cell loss was observed in the group exposed to 5 μM Aβ after incubation in comparison to control group ($p<0.001$). Fasudil was found to prevent cell loss considerably in the group treated with Fasudil 2.5 μM and Aβ compared to the group treated with Aβ alone ($p<0.001$).
Conclusion: In the present study, we found that Aβ caused abundant cell death in the murine C8-D1A astrocyte cell line and Fasudil effectively prevented cell loss induced by amyloid beta.
Key Words: *Amyloid beta; Fasudil; Alzheimer's disease; Astrocyte cell line*

INTRODUCTION

Alzheimer's disease (AD) is characterized by neuronal loss and degeneration of the central nervous system (CNS) and accounts for the majority of dementia. AD is the fourth leading cause of death in the western countries and affects more than 20 million globally. Therefore, elucidation +of the exact mechanism of neuronal cell death, development of agents that prevent neuronal loss and determination of novel strategies for prevention and treatment of the disease have become the major goals in AD [1].

Amyloid beta (Aβ) is a protein of 40-42 amino acids that are 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 [2]. APP is cleaved by proteolytic enzymes α, β and γ secretases and undergoes metabolism. Alpha secretase cleaves APP in between amino acid residues 16 and 17 and a new extracellular protein known as APP- α or sAPP is generated instead of soluble Aβ. Of the other two enzymes, β-secretase cleaves APP at the amino terminal and γ-secretase at the carboxy terminal, thereby amyloid β is produced. Resulting amyloid β peptides are 40-42 amino acid long and 42-amino acid Aβ fragments are more amyloidogenic and precipitate first [3]. Subsequently, amyloid β aggregates in the form of diffuse plaques and evolves into dense neuritic plaques [4].

Since pathological findings of AD involve amyloid plaques and neurofibrillary tangles in the brain as well as astroglial and microglial activation and the presence of various neuroinflammatory mediators including cytokines, acute phase proteins and proteases around the plaques suggest that inflammatory process and glial activation contribute to the progression of AD [5]. Toxicity associated with accumulation of Aβ on the neurons induces activation of several proinflammatory cytokines like TNF-alfa and interleukins in microglia and astrocytes, resulting in pro-apoptotic and synaptotoxic effects [6]. Astrocytes were first described by Rudolf Virchow in 1856 as cells that support neuronal function both metabolically and structurally [7]. Astrocytes are the predominant cell type in the brain [8] and comprise 80% of the glial cells [9]. Astrocytes have a key role in the breakdown and clearance of Aβ, provide trophic support to neurons and form a protective barrier between Aβ deposits and neurons [10]. Rho is a small G protein and has many functions at the cellular level including cell proliferation, adhesion and apoptosis. Rho kinase is the most commonly recognized and studied downstream effector of Rho and has two isoforms: ROCKα/ROCK II and ROCKβ/ROCK I [11]. Apoptosis or programmed cell death is a physiological process in which cells that have lost their biological function and are no longer needed are set to die without harming their environment. Both isoforms of ROCK are known to have an important role in apoptosis. While ROCK I is activated by caspase- 3 mediated cleavage [12], ROCK II is activated by granzyme B-mediated cleavage [13]. In the cell, Rho has an active GTP-bound form and an inactive GDP-bound form [14]. Rho proteins act as molecular switches in the signaling pathways that control gene expression and actin cytoskeleton formation. Rho-kinase is believed to be involved in the regulation of stress fiber formation, vascular smooth muscle contraction, cell migration and proliferation [15]. Additionally, recent evidence for the role of ROCK in the regulation of apoptosis led to an increase in *in vitro* and *in vivo* research studies [16]. The neuroprotective mechanism of ROCK inhibition is associated with increased regional cerebral blood flow, reduced inflammatory response, altered polymerization of neuronal actin cytoskeleton, tau hyperphosphorylation and increased p25/CDK5 [17]. However, few studies have explored the effects of ROCK on neuronal apoptosis following cerebral ischemia. Recent studies demonstrated the causative role of ROCK in ischemic injury [18]. There are several agents that inhibit Rho-kinase including Y-27632, Fasudil (HA-1077), Hydroxyfasudil, Y-32885, Y-39983, H-1152P, SLx-2119, azabenzimidazole-aminofurazans (GSK-269962A and SB-772077) and Wf-536 [18]. Recently, Fasudil was shown to protect brain tissue against ischemic injury [19]. Initially classified as an intracellular calcium antagonist, Fasudil has been used in Japan for a while for the treatment of cerebral vasospasms after subarachnoid hemorrhage [20]. The mechanisms involved in Fasudil's actions against cerebrovascular occlusion are thought to include increased cerebral blood flow and reduced inflammatory response [19,21]. Decreased inflammatory response induced by ROCK

[1]*Department of Medical Biochemistry, Faculty of Medicine, Gaziantep University, Gaziantep, Turkey*

[2]*Department of Medical Pharmacology, Faculty of Medicine, SANKO University, Gaziantep, Turkey*

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Correspondence: Benlier N, Department of Medical Pharmacology, Faculty of Medicine, SANKO University, Gaziantep, Turkey. Telephone +918886042417, e-mail nbenlier@hotmail.com

blockage occurs via inhibition of neutrophil migration [21]. However, there is no evidence from previous studies suggesting a direct association of ROCK activity and neuronal cell injury during *in vivo* ischemic episodes.

In the present study, we aimed to evaluate cell viability in an astrocyte cell line treated with amyloid beta peptide and fasudil.

MATERIALS AND METHODS

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. C8-D1A astrocytes grow in a liquid medium known as Dulbecco's Modified Eagle's Medium (DMEM). The astrocyte C8-D1A cell line was incubated in the DMEM (complete medium) containing 10% inactivated fetal bovine serum (FBS) and 1% penicillin/streptomycin in a sterile incubator at 37°C with 5% CO2 and 95% air. To allow cell growth, approximately 1×10^6/ml cells were transferred to a 75 cm^2 sterile culture flask (T75, Thermo) containing 15 ml of the complete medium and left to incubate for three days. Cultured cells passaged at a split rate of 1:2 every 2 or 3 days. Trypsin/EDTA (Sigma) was used during passages. Initially, the complete medium and Trypsin/EDTA were kept at water bath at 37°C for 15 minutes, then transferred to the safety cabinet and all of the medium contained in the flask was discarded. Thus, cells were cleared of the overlying medium. 3 ml of Trypsin/EDTA was added on the cells that attached to the bottom of the flask and kept in the incubator for 5 minutes. After 5 minutes, cells were taken out from the incubator and examined under the light microscope. It was observed that the cells were detached from the bottom of the flask. Afterwards, the cells were transferred to the safety cabinet and completed to 10 ml by adding 7 ml of the complete medium. The entire solution was transferred to a 15 ml Eppendorf tube and centrifuged at 120 relative centrifugal force (RCF) for 5 minutes. Pellet formation was observed at the bottom of the tube after centrifugation. Then the supernatant medium was discarded, and the cell pellet was taken using fingers. 10 ml of the complete medium was added to separate and disperse the cells and the cells were aspirated by pipetting. After sufficient pipetting, 5 ml of the solution was transferred into each of the two 75 cm^2 flasks. Then, 10 ml of the complete medium was added, and flasks were left to incubate.

Dimethyl Sulfoxide (DMSO) was used for cryopreservation. The cells contained in two flasks were removed with Trypsin/EDTA and then transferred into two separate 15 ml Eppendorf tubes and centrifuged for 5 minutes at 120 RCF. At the same time, 6 ml of the complete medium was transferred into an autoclaved 15 ml Eppendorf tube and 5% DMSO (0.3 ml) was added to obtain a solution. Following centrifugation, the medium in the tubes were discarded and the cell pellet was agitated. The entire content of DMSO + complete medium solution was transferred into the first tube and all cells were aspirated by pipetting and transferred into the second tube. Cells in the second tube were pipetted to obtain a cell suspension. The cells were divided into four separate 1.8 ml tubes, each containing 1.5 ml of cells. Tubes were labelled with the cell identity, passage number, date and name and stored at -80°C. Two days later, the tubes were transferred for stock cryopreservation at -152°C.

MTT viability test

MTT reduction method relies on the ability of viable cells to reduce a soluble tetrazolium salt [3-(4,5-dimethyl-2-thiazolyl)- 2.5-diphenyl-2H-tetrazolium bromide] (MTT) by mitochondrial succinate dehydrogenase activity of viable cells. As a result of degradation of the tetrazolium ring, pale yellow MTT dye is reduced to a dark blue-purple formazan product and the zones containing viable cells with intact mitochondrial function are stained purple [5,23,28]. Initially, cells were inoculated into a microtiter plate such that each of the 96 wells contained 25×10^3 of cells and left to incubate for 24 hours at 37°C. Then, dose titration was performed for Aβ (2.5 µM, 5 µM, 10 µM, 15 µM, 20 µM, 40 µM, 80 µM) and plates were placed in the incubator in groups of six including control group for 24 hours. After 24 hours, 100 µl of MTT solution (final concentration, 1 mg/ml stock solution at ambient conditions) was added into each well and incubated for 1.5 hours at 37°C. Following incubation, MTT solution was removed from the wells and 100 µl of 2-propanol was added into the wells and reincubated for another 1.5 hours. At the end of 1.5 hours, the microplate was read on a microplate reader at a wavelength of 570 nm to obtain absorbance values. 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.

Inverted microscope

Astrocyte cells were exposed to Fasudil (2.5 µM) and then Aβ (5 µM) for 24 hours. Subsequently, photographs of the resulting cell samples were taken with 40X magnification objective of the inverted microscope.

Statistical analyses

Data obtained from the study were analyzed with the SPSS 20 (Statistical Package for Social Sciences) software using one-way ANOVA analysis of variance. Statistical differences between the groups were assessed using Tukey's test. A p value equal to or less than 0.05 was considered statistically significant. For descriptive statistics, mean ± standard deviation (SD) was used.

RESULTS

The present study had an "independent samples" design and a statistically significant difference was found between the percentage values of three separate groups (p=0.001). Then, pairwise comparisons were conducted to determine the groups showing significant difference and a statistically significant difference was found between Fasudil 2.5 µM and Amyloid beta 5 µM (p=0.001). Similarly, a statistically significant difference was observed between Fasudil+Amyloid beta and Amyloid beta 5 µM (p=0.001). Finally, Fasudil+Amyloid beta and Fasudil 2.5 µM were compared and no statistically significant difference was found (p=0.952) (Table 1).

Table 1: Significance levels determined by Tukey's test of cell viability (for each pairwise comparison).

Group I	Group II	Mean Difference (I versus II)	P	95% confidence interval
	Amyloid beta 5 µM	60.700	0.001*	(35.723- 85.678)
Fasudil 2.5 µM	Fasudil + Amyloid beta	27.833	0.952	(-22.193 VB B- 27.760)
	Fasudil 2.5 µM	-60.700	0.001*	(-85.678 - 35.723)
Amyloid beta 5 µM	Fasudil + Amyloid beta	-57.917*	0.001*	(-81.466 - 34.368)
Fasudil+Amyloid beta	Fasudil 2.5 µM	-27.833	0.952	(-27.760 - 22.193)

Amyloid beta 5 µM	57.917*		0.001*	(34.368 - 81.466)

* P<0.05: Statistically significant.

Effects of varying concentrations of Aβ on cell viability

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 increase in cell loss was observed in the group exposed to 5 µM Aβ after incubation in comparison to control group ($p<0.001$). Throughout the study, 5 µM concentration of Aβ was used to induce stress in the cells. The cell viability of untreated cells (control) was set to 100% and the following percent viability values were observed: 2.5 µM: 20.10; 5 µM: 18.68; 10 µM: 22.34; 20 µM: 22.63; 40 µM: 23.41; 80 µM: 22.43 (Figure 1).

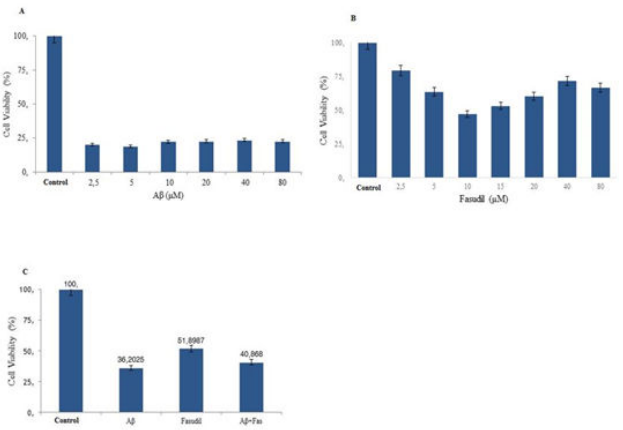

Figure 1) A. Effects of varying concentrations of Aβ on cell viability. B. Effects of varying concentrations of fusodil on cell viability. c. Effects of Aβ (5 µM) and Fasudil(2.5 µM) on cell viability.

Effects of varying concentrations of Fasudil on cell viability

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. As a result, a considerable decline in cell viability was found in the group exposed to 2.5 µM Fasudil after 24-hour incubation versus control group ($p<0.001$). The cell viability of control was set to 100% and the following percent viability values were observed: 2.5 µM: 79.38; 5 µM: 63.7; 10 µM: 42.27; 15 µM: 53.32; 20 µM: 60.50; 40 µM: 71.72; 80 µM: 66.69. 10 µM Fasudil-treated group displayed the greatest cell loss and 2.5 µM Fasudil-treated group had the least cell loss.

Fasudil was found to prevent cell loss considerably in the group treated with Fasudil 2.5 µM and Aβ compared to the group treated with Aβ alone (76.60 ± 18.14%) ($p<0.001$). The cell viability of control was set to 100% and the following percent viability values were observed: approximately 36.20 for Aβ 5 µM, approximately 51.89 for Fasudil 2.5 µM and approximately 40.86 for Aβ+Fasudil.

Morphological changes induced by Fasudil in astrocytes treated with Aβ

Astrocytes were exposed to Fasudil (2.5 µM) and then Aβ (5 µM) for 24 hours. Photographs of the resulting cell samples taken with 40X magnification objective of the inverted microscope showed a marked increase in cell death in Aβ-treated group (Figure 2) in comparison to control group (Figure 2). Also, a lesser degree of cell death was observed with Fasudil treatment alone (Figure 2) but Fasudil substantially reduced cell death caused by Aβ (Figure 2).

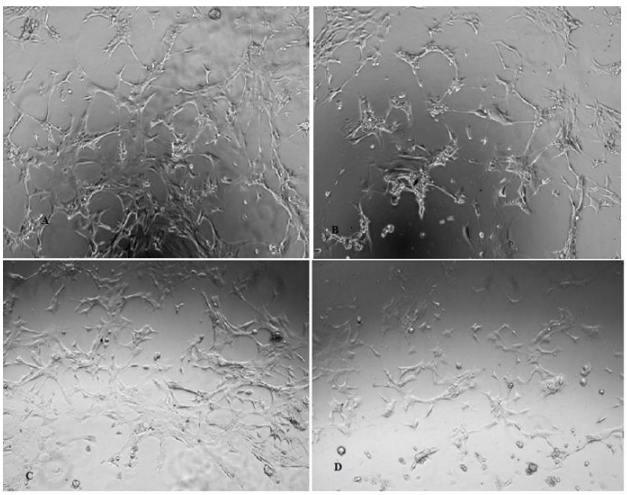

Figure 2) A. Microscopic images of control astrocyte group obtained at 24 hours (40X Magnification). B. Microscopic images of astrocytes treated with Aβ (5 µM) obtained at 24 hours. C. Microscopic images of astrocytes treated with Fasudil (2.5 µM) obtained at 24 hours. D. Microscopic images of astrocytes treated with Aβ and Fasudil obtained at 24 hours.

DISCUSSION

Alzheimer's disease (AD) is a neurodegenerative disease and the most common form of dementia in the elderly. There has been a rapid rise in the incidence of Alzheimer's disease and currently AD ranks fourth as a cause of mortality in developed countries [1]. Shared characteristics of the brains with Alzheimer's disease include senile plaques, amyloid plaques caused by accumulation of beta-amyloid and neurofibrillary tangles composed of aggregates of hyperphosphorylated tau protein.

Rho kinase (ROCK) belongs to a family of serine/threonine kinases that are activated via interaction with Rho GTPases and has two isoforms, namely ROCK1 and ROCK2 [11]. 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 [12-15]. ROCK inhibition helps prevent neuroinflammation and further neuronal damage.

Fasudil is a potent ROCK inhibitor. In the current study, we intended to investigate cell viability following treatment of murine astrocyte cells with Aβ and Fasudil.

Astrocytes constitute 30 to 50% of human neural cells [8,9] and they have long been considered to provide trophic and metabolic support to neurons. Astrogliosis is associated with a wide range of cellular events including the release of nitric oxide (NO), reactive oxygen species, proinflammatory cytokines (e.g., TNF-alpha, IL-1β and IL-6) and prostaglandins. Recent studies have demonstrated in AD cell models that astrocytes are activated by Aβ and this in turn, leads to release of inflammatory factors that impair synaptic and neuronal health [5,22,23]. Rather than being simply a "bystander pathology", experimental evidence demonstrates that

neuroinflammation plays an active role in the development and progression of AD [24]. Aβ is known to activate astrocytes and microglia via induction of inflammatory signal transmission [22,23]. Both microglia and astrocytes release numerous pro- and anti-inflammatory cytokines under different conditions. Changes in the cellular environment elicit a range of responses that are not well defined or predictable and that likely account for reports of both protective and neurotoxic effects of glial stimulation in models of disease [23,25].

In the present study, cell viability checks following 24-hour incubation of astrocytes treated with 5 μM Aβ, significant cell death was observed in the Aβ-treated group versus control group but cell loss was significantly reduced in the group exposed to 2.5 μM Fasudil in addition to Aβ. 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) [26].

CONCLUSION

In conclusion, we found that Aβ caused abundant cell death in the murine C8-D1A astrocyte cell line and Fasudil effectively prevented cell loss induced by amyloid beta. Dose and time-related changes in responses to Aβ and Fasudil treatment in the murine C8-D1A astrocyte cell line and protective effect of 2.5 μM Fasudil against amyloid beta toxicity were shown. Differential cell viability responses following exposure of the cell line to Fasudil and Aβ as observed in this study may contribute to better understanding of the pathogenesis of neurodegenerative diseases such as Alzheimer's disease and development of novel treatment options.

CONFLICT OF INTEREST

The authors declare that they have no competing interests.

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