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Oligomeric Tau-induced oxidative damage and functional alterations in cerebral endothelial cells: Role of RhoA/ROCK signaling pathway

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Abstract

Despite of yet unknown mechanism, microvascular deposition of oligomeric Tau (oTau) has been implicated in alteration of the Blood-Brain Barrier (BBB) function in Alzheimer's disease (AD) brains. In this study, we employed an *in vitro* BBB model using primary mouse cerebral endothelial cells (CECs) to investigate the mechanism underlying the effects of oTau on BBB function. We found that exposing CECs to oTau induced oxidative stress through NADPH oxidase, increased oxidative damage to proteins, decreased proteasome activity, and expressions of tight junction (TJ) proteins including occludin, zonula occludens-1 (ZO-1) and claudin-5. These effects were suppressed by the pretreatment with Fasudil, a RhoA/ROCK signaling inhibitor. Consistent with the biochemical alterations, we found that exposing the basolateral side of CECs to oTau in the BBB model disrupted the integrity of the BBB, as indicated by an increase in FITC-dextran transport across the model, and a decrease in trans endothelial electrical resistance (TEER). oTau also increased the transmigration of peripheral blood mononuclear cells (PBMCs) in the BBB model. These functional alterations in the BBB induced by oTau were also suppressed by Fasudil. Taken together, our findings suggest that targeting the RhoA/ROCK pathway can be a potential therapeutic strategy to maintain BBB function in AD.

Keywords

Oligomeric Tau; Blood-brain barrier; Endothelial permeability; Tight junction proteins; RhoA/ROCK signaling; Protein oxidation; Oxidative stress

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

CRediT authorship contribution statement

Faruk Hossen: Writing – original draft, Methodology, Investigation, Formal analysis, Data curation. **Grace Y. Sun:** Writing – review & editing. **James C. Lee:** Writing – review & editing, Supervision, Funding acquisition, Conceptualization.

Ethics approval and consent

All animal experiments were performed in accordance with the approved animal protocols and guidelines established by University of Illinois at Chicago.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.freeradbiomed.2024.05.044.

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1. Introduction

The Blood-Brain Barrier (BBB) is known to serve as a critical interface between the systemic circulation and the central nervous system (CNS), and thus playing a pivotal role in regulating the transport of nutrients, metabolic waste products, and neurotoxic substances in and out of the brain [1,2]. Dysfunction of the BBB has been increasingly recognized as a contributing factor for the pathogenesis and progression of numerous neurodegenerative diseases [3,4]. Recent investigations have unveiled a significant association between BBB leakage and a range of neurological disorders including AD [3,5–7]. BBB dysfunction facilitates the infiltration of peripheral immune cells and inflammatory mediators into the CNS, triggering a cascade of neuroinflammatory responses [8–10]. Dysfunction of the BBB impairs the clearance of amyloid-beta (Aβ) peptides, tau oligomers, and other protein aggregates associated with neurodegeneration [11–14]. Accumulation of these toxic species in the brain can further exacerbate neuronal dysfunction and degeneration due to increased oxidatively damage of proteins and generation of free radicals [15–19].

Tau is recognized as a proteo-toxic protein because it induces neuronal damage by disrupting ribosomal function and thus attenuating protein synthesis [20,21]. Particularly, the oligomeric forms of tau can induce oxidative damage and disrupt endothelial cell function, leading to increased BBB permeability [22–25]. This toxic oligomeric peptide can modify the endothelial characteristics of the BBB and disturb the integrity of tight junctions between vascular endothelial cells [22,23,26, 27]. This disruption allows the entry of neurotoxic molecules, inflammatory mediators, and potentially harmful substances from the bloodstream into the brain parenchyma and exacerbates neurodegeneration [28,29]. Proteins that are oxidatively modified by oTau may induce structural changes within the cell and can lead to functional impairments such as interfering cellular functions, protein expression and gene regulation, modulation of protein turnover, and cell signaling pathways [30–37].

The proteasome responsible for removing oxidatively modified proteins from cells can play a vital role in maintaining cellular homeostasis [38,39]. Recent studies have demonstrated a decline in proteasome activity in the AD brains, which correlates with elevated levels of oxidatively modified/misfolded proteins, aggregated amyloid beta (Aβ), hyperphosphorylated Tau, and neurofibrillary tangles (NFTs) [40–44]. Reduced proteasomal activity has been shown to increase Tau accumulation, thus contributing to neurotoxicity and cognitive dysfunction in both cellular and animal models of neurodegenerative disorders [45–48]. However, the mechanism through which oTau affects proteasomal activity have yet to be fully elucidated.

RhoA and its downstream effector, Rho-dependent coiled-coil kinase (ROCK), belong to the GTPase members of the Rho subfamily [49]. Together, the RhoA/ROCK pathway regulates a wide array of cellular functions, including cellular polarity, motility, adhesion, proliferation, contraction, and migration [50,51]. This signaling pathway is also known to regulate cytoskeletal dynamics, cell contraction, and permeability function in endothelial cells [51,52]. Dysregulation of this pathway has been implicated in various vascular and

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neurodegenerative disorders [53–55]. Numerous studies have indicated that this signaling pathway involved in Oxidative Stress, alteration of endothelial cell function and BBB disruption [56–59]. Recent investigations have indicated the implication of the RhoA/ROCK signaling pathway in AD pathology [53,55,60]. This signaling pathway elicits phagocytosis and fosters neuroinflammation in neuronal cells and plays crucial role in neuronal disease development and progression [61,62].

In this study, we explored the impacts of oTau on protein oxidation and proteasome activity within the CECs and linking cerebral endothelial permeability to the RhoA/ROCK pathway. With this *in vitro* BBB model, we investigated dextran permeability activity, transendothelial electrical resistance (TEER), and used an immune cell transmigration assay to quantify endothelial permeability following oTau treatment of CECs. Exposure of CECs to oTau resulted in increase in protein oxidation, a reduction in TJ protein expressions, and subsequently alterations in cerebral endothelial permeability. Notably, these effects were effectively mitigated by the inhibition of RhoA/ROCK signaling.

2. Materials and methods

2.1. Materials

Tau-441 were purchased from rPeptide (Bogart, GA), Fasudil and Heparin were purchased from Stem Cell Technologies (Cambridge, MA). Primary antibodies and reagents were purchased as follows: Anti-Tau (T22) from Millipore Sigma (St. Louis, MO), Anti-occludin, ZO-1 and claudin-5, CD31 (PECAM-1) Monoclonal Antibody (390), p47phox and p-p47phox antibody from Thermo Fisher Scientific (Waltham, MA), Myosin Light Chain 2 and Phospho-Myosin Light Chain 2 (Thr18/Ser19) were purchased from Cell Signaling (Beverly, MA). APC conjugated CD45 antibody was purchased from BioLegend (San Diego, CA). Ficol Plus was purchased from Cytiva (Marlborough, MA). Protein Carbonyl Assay kit and Proteasome 20S activity assay kit was purchased from Abcam (Waltham, MA). EBM-2 Endothelial basal medium (Lonza, Basel), Cell culture inserts from (Corning, NY), cOmplete protease inhibitor cocktail, PhosSTOP phosphatase inhibitor cocktail, β-mercaptoethanol (β-ME), and Triton X-100, Puromycin and FITC-Dextran were purchased from Millipore Sigma (St. Louis, MO). Dulbecco's phosphate-buffered saline (DPBS) were purchased from Life Technologies (Grand Island, NY). Radioimmunoprecipitation assay buffer (RIPA buffer), BCA protein assay kit, ProLong™ diamond antifade mountant with DAPI, SuperSignal™ west femto maximum sensitivity substrate, SuperSignal™ west pico plus chemiluminescent substrate, and Restore™ PLUS western-blot stripping buffer were purchased from Thermo Fisher Scientific (Waltham, MA). Laemmli sample buffer and tris buffered saline (TBS) were purchased from Bio-Rad (Hercules, CA). Antibodies, reagents, and assay kits used in this study, their details information (company name, catalog number, etc.) are provided in supplementary Tables 1 and 2

2.2. Animals

C57BL/6 mice were obtained from Charles River (Wilmington, MA). These mice were housed under a 12h light/dark cycle and temperature-controlled conditions, and access to food and water. All experiments were conducted in compliance with approved animal

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protocols and guidelines established by the Institutional Animal Care and Use Committee (IACUC) at the University of Illinois at Chicago.

2.3. Oligomeric Tau preparation and characterization

The preparation of oligomeric Tau followed the protocol outlined in Ref. [63]. Briefly, Tau-441 was resuspended at a concentration of 1 mg/mL in a solution (50 mM MES, 100 mM NaCl, 0.5 mM EGTA, pH 6.8). Oligomerization of Tau was induced using 0.2 % heparin in PBS incubated for 3h at 37 °C in a CO_2 incubator. oTau was characterized by Western blot analysis and atomic force microscopy (AFM) imaging.

2.4. Isolation and culture of primary mouse CECs

Primary mice cerebral endothelial cells (CECs) were isolated from C57BL/6 mice as described previously [64]. Detailed methodology and cell characterization can be found in the Supplementary file (Primary mouse CECs Isolation, Culture and Characterization).

To investigate the effects of oTau on CECs, cells were serum starved with EBM-2 Endothelial basal media for 3h. Subsequently, they were treated with oTau (0.2 μM) for 30 min or 24h, depending on specific experimental conditions. To investigate whether the RhoA/ROCK signaling pathway was involved in the effects of oTau on CECs, cells were pre-treated with Fasudil (50 μM), the RhoA/ROCK signaling inhibitor for 1 h prior to oTau treatment.

2.5. Western Blotting

Cells were cultured in 35 mm dishes until they reached 80–90 % confluence. Prior to treatment, cells were starved with serum-free media for 3 h. Subsequently, cells were pre-treated with Fasudil for 1 h and then treated with oTau for 24 h for assessment of total proteins and 30 min for phosphorylated proteins. For the assessment of phosphorylated proteins, only 30 min of treatment oTau was sufficient. After treatment, the CECs were thoroughly rinsed with PBS and subsequently lysed with ice-cold RIPA buffer supplemented with the cocktail of protease and phosphatase inhibitors. Protein concentration in cell lysate was determined using a BCA assay. For gel electrophoresis, equivalent quantities of protein from each sample were resolved using SDS-polyacrylamide gels for electrophoresis. After electrophoresis, the gels were overlaid with methanol-activated polyvinylidene difluoride (PVDF) membranes for transferring proteins from gel to the membrane. Subsequently, the PVDF membranes were washed with wash buffer then blocked using blocking buffer for 1 h at room temperature. Next, the membrane was subjected to overnight incubation with primary antibodies at 4 °C, followed by a 1 h incubation with secondary antibodies conjugated with horseradish peroxidase (HRP) at room temperature. Signal detection was accomplished using the SuperSignal™ West Pico Plus chemiluminescent substrate and images were captured using a myECL imager. When necessary, a stripping buffer was applied to remove bound antibodies at room temperature for a period of 15 min. Subsequently, the samples were reblotted utilizing the same procedures as described above. Each experiment was repeated at least three times. In the experiment for detection of occludin, ZO-1 and claudin-5, the membrane was reblotted with antibodies for β-actin as protein loading control. However, in experiments in which short incubation time (30 min)

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was used to detect phosphorylated proteins, we observed consistency in loading of the non-phosphorylated protein. Therefore, the total non-phosphorylated protein also served as loading control. Details on source of antibodies for phospho- and non-phospho-proteins are listed in the supplementary Table 1.

2.6. Protein Carbonyl Assay (western blot)

To assess the presence of carbonyl groups resulting from oxidative reactions within proteins, a protein carbonyl Western blot assay was performed. The quantification of protein carbonyl groups was conducted following the guidelines provided by the manufacturer. Details on source of this assay kit is listed in the supplementary Table 2.

2.7. Proteasome activity assay

The assessment of proteasome activity was carried out using total protein lysates employing a Proteasome 20S Activity Assay kit, adhering to the instructions provided by the manufacturer. Briefly, this kit contains a homogeneous fluorescent assay system that measures the chymotrypsin-like protease activity associated with the proteasome complex in cultured cells. It uses LLVY-R110 as a fluorogenic indicator for proteasome activities. Cleavage of LLVY-R110 by the proteasome generates strongly green fluorescent which can be monitored fluorometrically. The fluorescence intensity was quantified at excitation/emission wavelengths of 490/525 nm, utilizing a fluorescent microplate reader. Details on source of this assay kit is listed in the supplementary Table 2.

2.8. Development of an in vitro BBB model

We followed the methodology outlined in our previous article to develop an *in vitro* BBB model [65]. Briefly, in this study, CECs were cultured on the membrane with 3.0 μm pore size of transwell inserts in 12-well plate for 9 days (until confluent). An endothelial volt/ohm meter for TEER-EVOM2 (World Precision Instruments, Sarasota, FL) was used to measure the TEER value. The TEER values were obtained by transferring the transwell inserts into the Endohm chamber. The concentric pairs of electrodes above and beneath the membrane caused a coincident current density flow across the membrane, and EVOM2 offered the transmembrane electrical resistance according to the current. All TEER values were determined after background subtraction with those of the insert membrane without cells. The validation of the BBB model was achieved when the TEER value increased to reach a maximum value and plateau as shown as supplementary Fig 1. The TEER value can be applied as a measure for the integrity of the BBB model. Details of the TEER protocol to measure electrical resistance is shown in Supplementary Fig. 2A.

2.9. Dextran assay for measurement of cell permeability

We adapted the dextran assay protocol for endothelial cell permeability measurement as previously described [65]. Briefly, the basolateral side of cells in the BBB model was treated with oTau for 24 h, followed by washing with 1x HBSS and then transferring to a fresh 24-well plate. Subsequently, 200 μL of HBSS containing 1 μg/mL FITC-Dextran was added to the insert chambers, while 600 μL of HBSS was added to the lower chambers. They were then placed in an incubator at 37 °C with 5 % CO_2, and gentle agitation was applied for 1h.

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After incubation, the concentration of FITC-Dextran that migrated to the lower chamber was determined. This measurement was carried out using a Microplate Reader, with excitation and emission wavelengths set at 492 nm and 520 nm, respectively. This approach enabled us to quantify the extent of endothelial permeability. The Dextran assay protocol to measure the passage of fluorescently labeled dextran molecules through endothelial cell monolayers is described in greater details in Supplementary Fig. 2B.

2.10. Isolation and characterization of Mouse Peripheral Blood Mononuclear Cells (PBMCs)

PBMCs were obtained from peripheral blood of healthy wild type C57BL/6 mice. The cells were isolated following a protocol described in Ref. [66] with minor modifications. The detailed protocol for isolating mouse PBMCs from peripheral whole blood can be found in supplementary file (Isolation of Mouse Peripheral Blood Mononuclear Cells) and schematic descriptions in the supplementary Fig. 3.

For characterization of PBMC, cells were washed with Flow Cytometry Staining Buffer (FACS) and aliquoted to a concentration of 1×10^6 cells/100 μL into FACS tubes. An appropriate amount APC conjugated primary antibody was added, and the mixture was vortexed. Cells were then incubated for 30 min on ice in the dark condition. Unbound antibodies were removed by washing the cells with FACS buffer and repeating this washing step two times. After vortexing, DAPI was added for nucleus staining, and the samples were prepared for flow cytometry analysis. Data from the flow cytometric analysis are provided in supplementary Fig. 4.

2.11. Trans endothelial migration assay of PBMCs in vitro BBB model

To investigate whether the RhoA/ROCK pathway was involved in the effects of oTau on the trans endothelial migration of PBMCs, CECs in the BBB model was pre-treated with Fasudil (50 μM) for 1 h, followed by treatment with oTau at the basolateral side for 24 h. After this time, ~10^6 PBMCs were then suspended in the apical side of the model and incubated for 24 h. The PBMCs that had transmigrated into the basolateral chamber of inserts were stained with APC conjugated anti mouse CD45 antibody and quantified using Nikon Eclipse Ti-E fluorescence microscope with a 20 × oil immersion objective lens.

2.12. Immunofluorescence labeling for occludin

For immunofluorescent labeling, cells were cultured on poly-D-lysine pre-coated coverslips in 6-well plate with complete growth medium and waited until they reached 80–90 % confluence. After the treatment, cells were washed with PBS and immediately fixed for 15 min with a 4 % paraformaldehyde solution. After the fixation, cells were washed with PBS and permeabilized with 0.1 % Triton X-100 for 5 min. To minimize non-specific bindings, cells were incubated with 5 % BSA in PBS for 1 h with gently agitation. Cells were then washed with PBS and incubated overnight at 4 °C with the anti-occludin antibody, followed by washing with PBS and incubation with Alexa Fluor 546-conjugated secondary antibody for 1 h at room temperature. Cells were further washed with PBS, and slides were mounted using ProLong Gold Antifade reagent containing 4′,6-diamidino-2-phenylindole to visualize

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nuclei. The occludin-staining was evaluated using a Nikon Eclipse Ti-E fluorescence microscope with a 20 × objective lens.

2.13. Data analysis

For most experiments, cells were cultured under the conditions stated above and measurements were conducted in triplicates. The results are expressed as mean ± standard deviation (SD). Statistical analysis involving multiple groups was performed using one-way analysis of variance (ANOVA), followed by Tukey's post hoc honestly significant difference (HSD) test, as executed in GraphPad Prism (version 8.10). Statistical significance was defined as p-values < 0.05.

3. Results

3.1. oTau activated the ROCK kinase activity in CECs

ROCK (Rho-kinase), an effector molecule of RhoA, which downstream of various extracellular signals in cells [67–69]. Numerous studies have shown that the ROCK is activated in various diseases including cardiovascular and neurodegenerative diseases [55,70–73]. The activated Rho-kinase phosphorylates the myosin light chain (MLC), which is involved in stress fiber formation, muscle contraction, and BBB disruption [74–78]. To determine whether oTau activates ROCK kinase activity in CECs, the expression of p-MLC and total MLC in CECs was measured by Western blot analysis. The results indicated that oTau increased the expression of p-MLC (Fig. 1). The elevated levels of p-MLC expression induced by oTau were suppressed when cells were pre-treated with Fasudil, a potent ROCK kinase inhibitor. Since ROCK activation leads to the phosphorylation of MLC in cells, this approach serves as a reliable indicator of ROCK activity in CECs.

3.2. oTau upregulated the phosphorylation of p47phox (subunit of NADPH oxidase) and increased protein oxidation

The p47phox is a vital subunit of NADPH oxidase which is primarily responsible for superoxide production through phosphorylation-driven mechanisms [79,80]. Here we tested if oTau could alter p47phox phosphorylation in CECs. Fig. 2A shows a marked increase in phosphorylated p47phox (p-p47phox) and Fig. 2B shows a significant increase in oxidative damage of proteins in CECs after treatment with oTau (0.2 μM). Surprisingly, the increases in p-p47phox and oxidatively damaged proteins were suppressed by pretreatment with Fasudil (50 μM), a ROCK inhibitor (Fig. 2A and B). These results suggest the role of RhoA/ROCK pathway in oTau-induced activation of NADPH oxidase and subsequent oxidative damage of proteins within the cells.

3.3. Effects of oTau on proteasome activity in CECs

Decrease in proteasome activity contributes to accumulation of the abnormally/oxidatively modified proteins in brain, and subsequently exacerbates disease conditions [81,82]. In our study, we found that exposing CECs to oTau led to a significant reduction of proteasome activity (Fig. 3). Pre-treating the cells with Fasudil (50 μM) also suppressed the reduction of proteasome activity induced by oTau (Fig. 3).

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3.4. oTau decreased tight junction (TJ) protein expressions in CECs

We further tested if oTau could impact the expression of TJ proteins in CECs. Cells were treated with oTau (0.2 μM) for 24 h and cell samples were subjected to Western blot analysis and immunofluorescence microscopy. Fig. 4 shows the effects of oTau to mediate a significant decrease in the expressions of occludin, ZO-1, and claudin-5 in CECs as compared to the control group. Downregulations of TJ proteins were totally abrogated by Fasudil (Fig. 4). Consistently, immunofluorescence microscopy of occludin in CECs also showed oTau mediated the decrease in occludin expression, which was counteracted by Fasudil (supplementary Fig. 5). These results suggests that oTau downregulated TJ proteins through the RhoA/ROCK pathway.

3.5. Effects of oTau on trans endothelial permeability

After exposing the basolateral side of the BBB model to oTau for 24 h, we characterized the paracellular permeability of the CEC layer using TEER and dextran assay. We found that oTau significantly decreased TEER (Fig. 5A) and increased the diffusion of dextran across the BBB model from the apical to basolateral side (Fig. 5B), and these changes were counteracted by pretreatment with Fasudil. These results suggest that the RhoA/ROCK pathway plays a role in oTau-induced increases in cerebral endothelial layer permeability.

3.6. oTau-induced transmigration of immune cells across the BBB

Fig. 6 shows oTau increased the transmigration of PBMCs, which was suppressed by the pretreatment with Fasudil, suggesting the role of RhoA/ROCK signaling pathway in oTau-enhanced migration of PBMCs across the endothelial layer.

4. Discussion

The BBB serves as a critical interface between the peripheral blood and the central nervous system, regulating exchange of molecules and transmigration of immune cells between the blood and brain [83,84]. Alterations of BBB function are among the earliest manifestations in different neurological disorders, including AD [85,86]. In fact, a leaky BBB has been observed in AD patients and AD mouse models [87–89].

While numerous studies have linked Aβ pathology to BBB disruption [90,91], the mechanism(s) underlying the role of oTau in BBB dysfunction remains largely unknown. Under pathological conditions such as AD, Tau becomes hyperphosphorylated and progressively forms aggregates ranging from small and soluble oligomeric Tau aggregates to helical filaments and eventually large, aggregated fibrils that compose the NFTs [92–100]. Abnormal protein aggregation, including oTau, is a crucial hallmark in AD pathology [101,102].

Oligomeric Tau (oTau), rather than NFTs, has been reported to be the most toxic form of Tau, altering cell metabolism and triggering neurodegeneration [24,25,103,104]. oTau is found in the brain and cerebrospinal fluid (CSF) of AD patients relatively early in the disease process and is a better correlate of cognitive impairment than NFT in mouse models of tauopathy [105–109]. Furthermore, neuron loss is evident before the appearance of NFT,

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showing that large fibrillar species of Tau are not the main drivers of neurodegeneration [104,110,111].

There is evidence that oTau can be hyperphosphorylated and released into the extracellular space and alters function of nearby neurons [112–114]. In fact, oTau is the major Tau species driving neurodegeneration in AD and other tauopathies [115–117]. Consequently, oTau is regarded the primary agent responsible for spreading Tau pathology from neurons to neurons throughout the brain [13,71,73–75]. Studies *in vitro* and in mouse models of tauopathy have shown that tau oligomers cause impaired axonal transport, synaptic and mitochondrial dysfunction, and, ultimately, neuronal death [27,117–121]. This species can also accumulate in endothelial and vascular smooth muscle cells in the cerebrovasculature in AD patients and in subjects with primary tauopathies, like progressive supranuclear palsy [23,122,123]. The broad range of cell types affected by oTau indicates a diverse array of pathogenic mechanisms, including neuron dysfunction and death, neuroinflammation from reactive astrocytes and microglia, and loss of cerebral blood flow and cerebrovascular dysregulation in AD and other tauopathies [124,125]. Moreover, Tau has been reported to play a role in mediating the increase of BBB permeability [22,122].

Pathological protein aggregates, such as Aβ and Tau, had a notable impact on oxidative stress with the production of superoxide and leading to protein oxidation in neuronal cells [15,16,126,127]. These effects can be demonstrated by increased phosphorylation of p47phox, a subunit of NADPH oxidase [80,128–130]. Therefore, increased phosphorylation of p47phox can be regarded as heightened oxidative stress [79,80] which likely contributes to oxidation of proteins, a process associated with cellular damage and dysfunction [129]. In this study, we demonstrated that inhibition of RhoA/ROCK by Fasudil could suppress oTau-induced phosphorylation of p47phox and oxidative damage of proteins, suggesting the involvement of the RhoA/ROCK pathway to regulate oxidative stress through NADPH oxidase.

The proteasome is an important machinery for removing oxidatively modified proteins from cells, thus playing a vital role in maintaining cellular homeostasis [38,131]. As proteins undergo oxidative damage, they can become misfolded or aggregated, rendering them non-functional and potentially toxic to the cell [132,133]. The proteasome thus acts like a cellular garbage disposal system, selectively recognizing and degrading the damaged proteins [38,134–136]. Proteasomal degradation-not only prevents the accumulation of harmful protein aggregates but also allows for the recycling of amino acids, thereby promoting cell health and longevity [137]. In this context, especially in neurodegenerative diseases like AD, where oxidative stress and protein aggregation are prevalent, proper functioning of proteasome is critical to counteract the accumulation of toxic protein species and maintain cell health [81,138]. However, dysregulation of the proteasome system is often observed in various neurological diseases, which can further exacerbate protein aggregation and cellular damage, making it an important focus of research in the field of neurodegenerative disorders [82,139–141]. Recent studies indicate that proteasome activity modulates amyloid toxicity, and inhibition of proteasome by Aβ could enhance amyloid and tau accumulation in AD [142–145]. Another recent study investigated the effects of Tau accumulation on proteasome function in a mouse model of tauopathy and found

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that the accumulation of insoluble Tau was associated with a decrease in the peptidase activity of brain 26S proteasomes [46]. Other studies found that Tau hyperphosphorylation and accumulation could also affect the proteasome activity [146,147]. In our study, we further provided additional understanding regarding the proteasome activity in AD by demonstrating the effects oTau in mediating proteasome activity through the RhoA/ROCK pathway.

In addition to biochemical studies, we also conducted functional studies for the impact of oTau on a BBB model using CECs. We demonstrated the role of RhoA/ROCK in oTau-induced downregulation of TJ protein expression and increase in cerebral CEC layer permeability to dextran as well as immune cells.

Rho-associated coiled-coil kinase (ROCK), a serine/threonine kinase regulated by the small GTPase RhoA, is known to play multiple roles in cell function, including cell migration, proliferation, and survival [148]. Many studies have shown that the RhoA/ROCK pathway is upregulated in AD brains [53,60]. Rho-associated protein kinases have been identified as therapeutic targets for vascular and parenchymal pathology in AD [149,150]. Under these conditions, RhoA/ROCK inhibitors have implicated in therapeutic potential against neurodegenerative diseases. A recent study showcased the remarkable potential of Fasudil in reversing neurodegenerative pathways in the brains of Alzheimer's model mice and substantial improvement was observed just two weeks of peripheral administration [151]. Another study found PT109B, 5-(1, 2-dithiolan-3-yl)-N-((1r,4r)-4-(isoquinolin-5-ylamino) cyclohexyl) pentanamide, a novel compound structurally related to Fasudil, could be a promising candidate for treating AD [152]. The RhoA/ROCK inhibitors have also demonstrated efficacy in reducing oligomeric tau protein, a hallmark of neurodegenerative diseases [153]. Furthermore, recent research has elucidated the role of Rho-associated kinases in regulating tau phosphorylation and amyloid metabolism. This study suggests that increased Rho kinase activity could disrupt synaptic plasticity, a mechanism that can be reversed by Rho-kinase inhibitors [154]. In another recent study, researchers conducted transcriptome analysis of hippocampal tissue from APPswe/PSEN1dE9 (APP/PS1, AD model) mice treated with Fasudil (ADF) and comparing changes with AD mice treated with saline (ADNS) and wild-type mice (WT) [155,156]. They discovered that several genes detrimental to brain health were significantly suppressed in Fasudil-treated mice, while genes beneficial for brain health were upregulated. Some recent findings also indicates that inhibition of Rho kinase could ameliorate cognition impairment and maintain the integrity of BBB [156,157]. Therefore, investigating how RhoA/ROCK mediates oTau-induced alteration in CEC function should further our understanding of the role of RhoA/ROCK in AD.

5. Conclusion

Our *in vitro* study suggests that oTau could activate the RhoA/ROCK signaling pathway in CECs, and subsequently alter the cerebral endothelial permeability (Fig. 7). Our findings should provide new insights into oTau-induced AD vasculopathy and offer additional evidence that regulation of the RhoA/ROCK pathway can be a potential therapeutic strategy to maintain the BBB integrity in AD. Future research should explore the *in*

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vivo relevance of these findings and investigate the translational potential of targeting the RhoA/ROCK pathway in AD. Preserving BBB integrity is a promising avenue for the development of therapeutic strategies to combat the progression of AD and potentially other neurodegenerative disorders characterized by BBB dysfunction.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Availability of data and materials

Data available on request. The data underlying this article will be shared on reasonable requests to the corresponding author.

List of Abbreviations

AD	Alzheimer's disease
APP	Amyloid precursor protein
BBB	Blood brain barrier
BSA	Bovine serum albumin
CECs	Cerebral Endothelial Cells
CNS	Central nervous system
CSF	Cerebral Spinal Fluid
DMEM	*Modified Eagle's Limiting Medium*
DMSO	Dimethyl sulfoxide
ELISA	Enzyme linked Immunosorbent Assay
NFTs	*Neurofibrillary Tangles*
oTau	Oligomeric Tau
PBS	Phosphate-buffered saline
ROCK	Rho-dependent coiled-coil kinase

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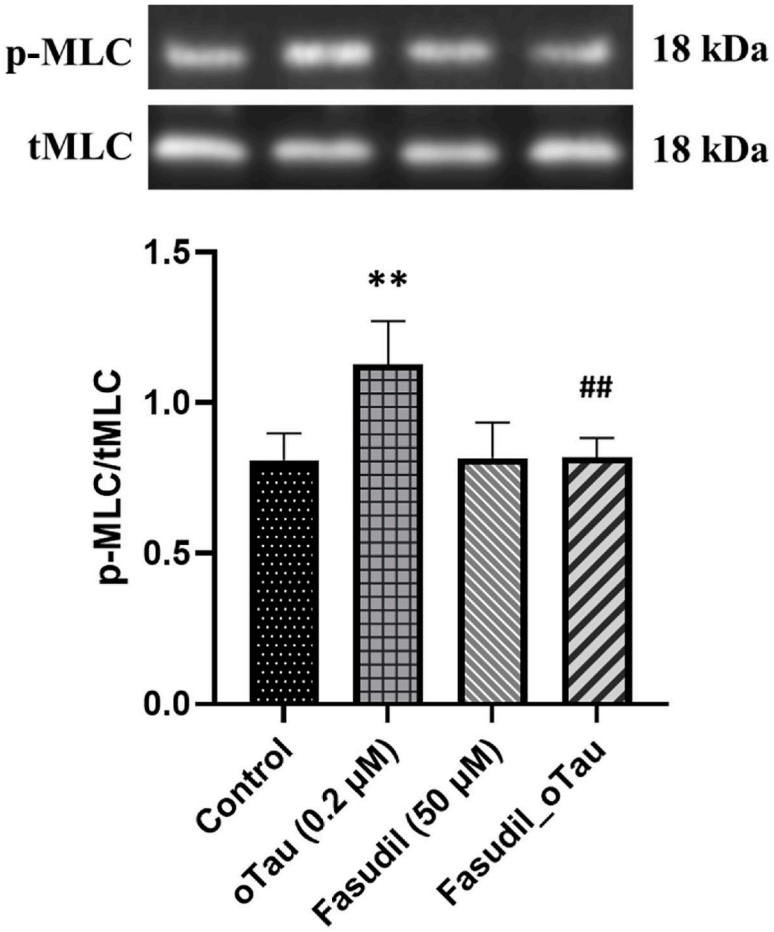

Fig. 1.

The Rho-associated kinase (ROCK) activity as determined in CECs. The expression of phosphorylation MLC (p-MLC) and total MLC (tMLC) in CECs was measured by Western blot analysis. ROCK activity is expressed as a relative blot expression ratio (p-MLC expression/tMLC expression). Data are presented as the mean ± SD and were analyzed by one-way ANOVA. $^{**}p < 0.01$ and $^{\#\#}p < 0.01$ compared with control and oTau treated group, respectively.

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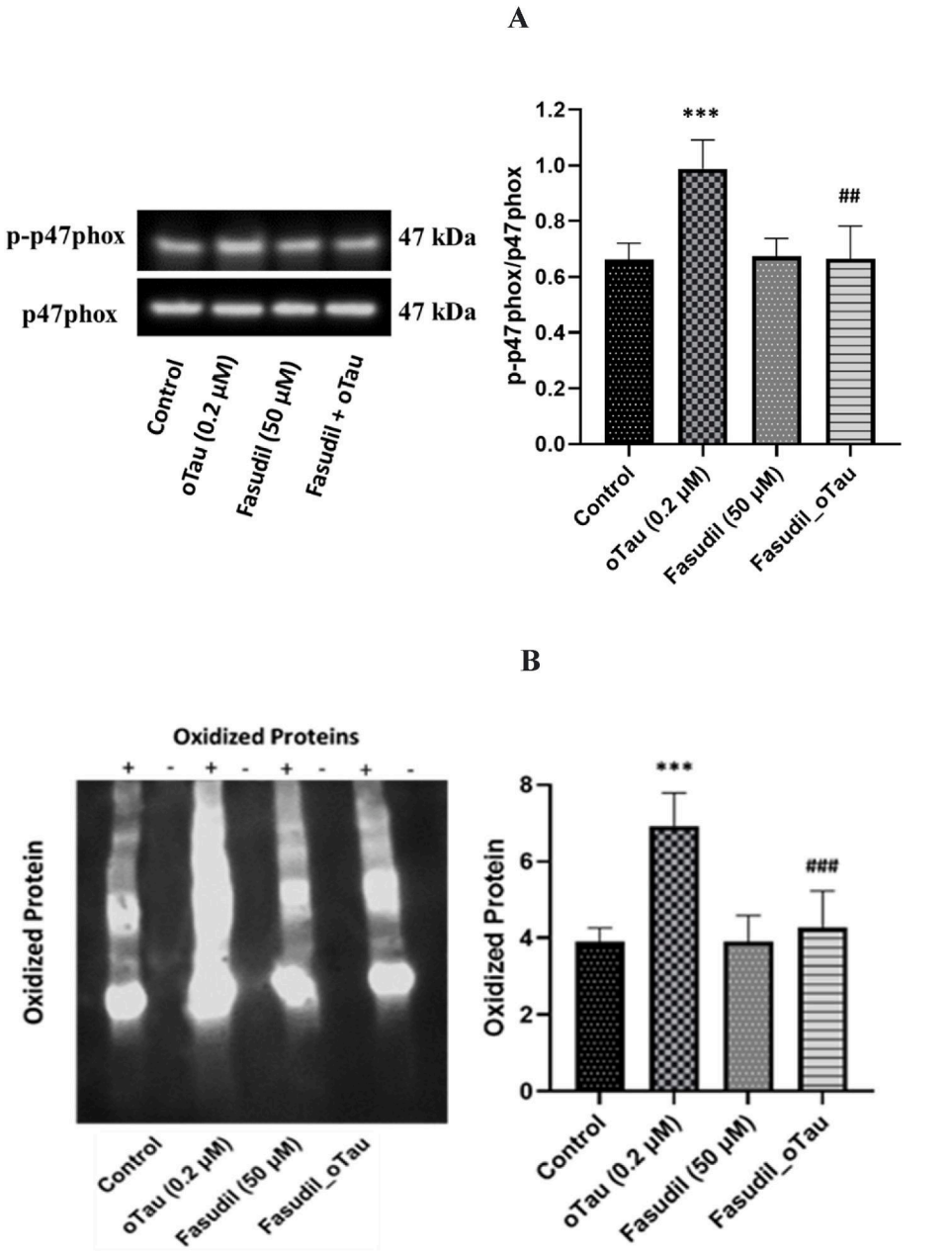

Fig. 2.

oTau increased p-p47phox expression and protein oxidation in CECs. Oxidized proteins were analyzed by the Protein Carbonyl Assay Kit (Western Blot). Each sample was loaded as a negative control (−) with non-derivatized procedure. Data are presented as the mean ± SD and were analyzed by one-way ANOVA. ***$p < 0.001$ and $^{###}p < 0.001$ compared with control and oTau treated group, respectively.

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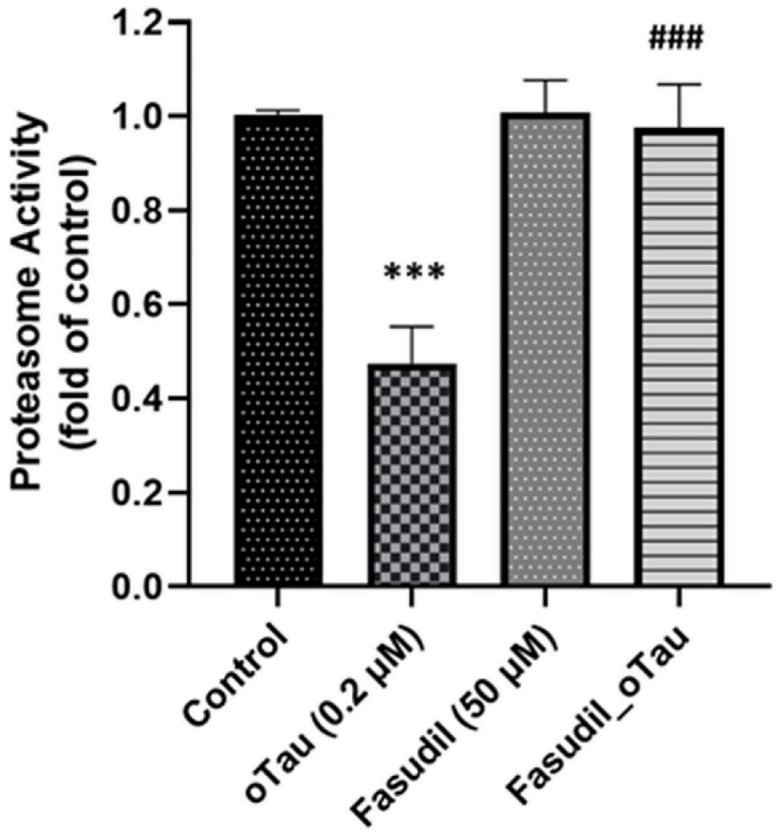

Fig. 3.

oTau decreased proteasome activity in CECs. Proteasome (20S) activity was analyzed using a colorimetric assay. The data are presented as the mean ± SD and were analyzed by one-way ANOVA. ***$p < 0.001$ and $^{###}p < 0.001$ compared with control and oTau treated group, respectively.

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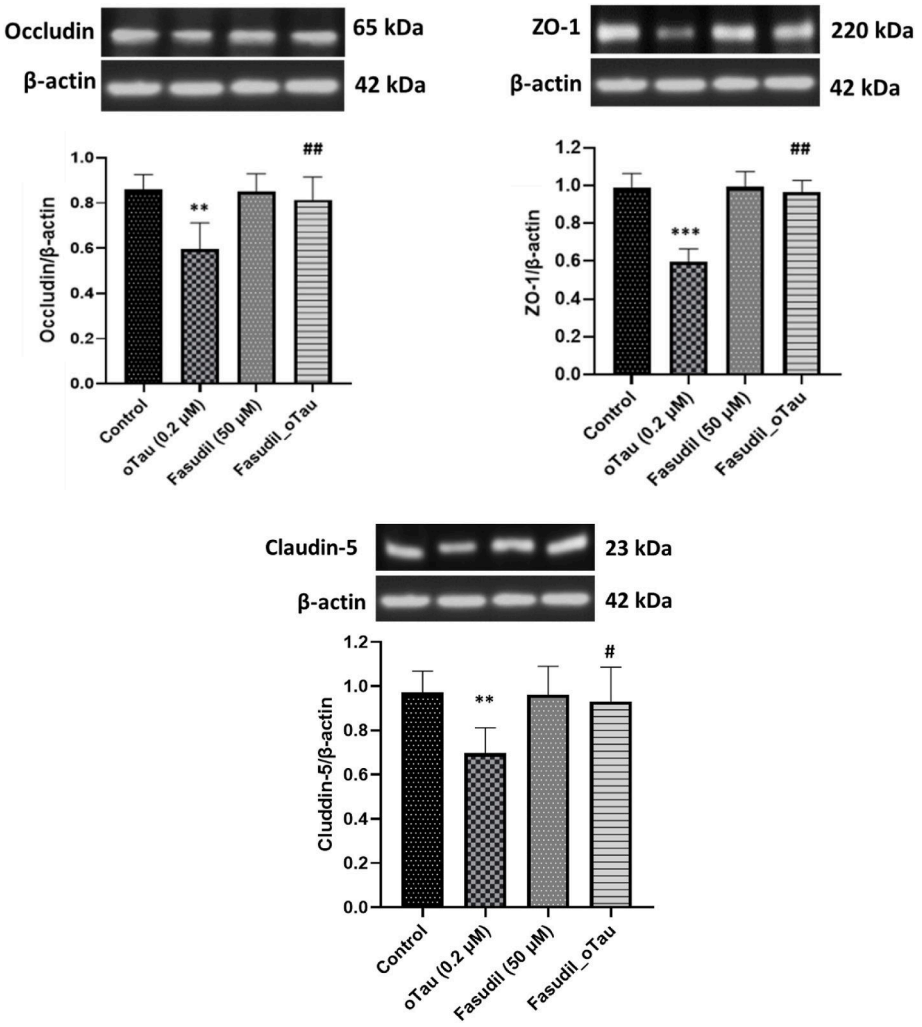

Fig. 4.

oTau decreased tight junction protein expressions in primary mouse CECs. Occludin, ZO-1 & Claudin-5 expression levels were analyzed by Western Blot assay. Data are presented as the mean ± SD and were analyzed by one-way ANOVA. ***$p < 0.001$, **$p < 0.01$, and $^{\#\#}p < 0.01$, $^{\#}p < 0.05$ compared with control and oTau treated group, respectively.

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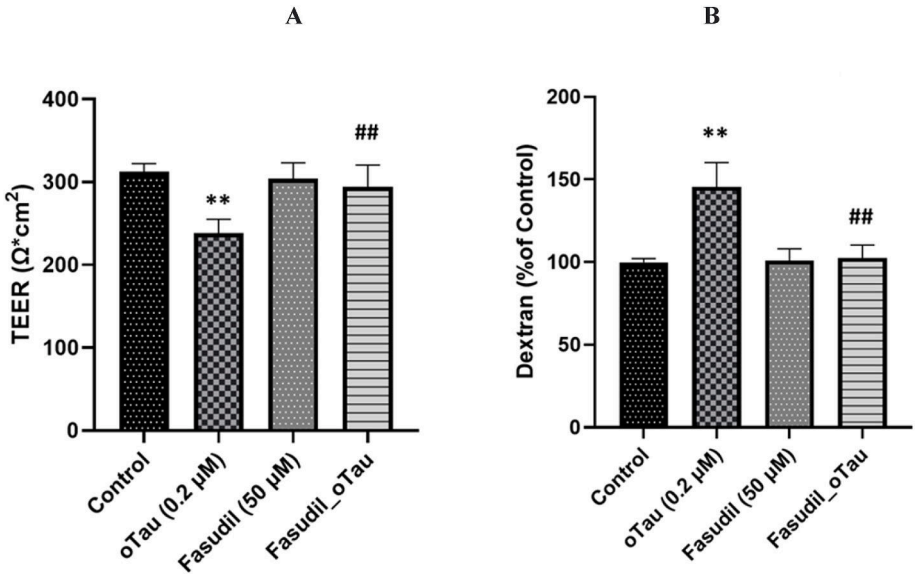

Fig. 5.

oTau altered the cerebral endothelial permeability as measured by TEER (A) and dextran assay (B). TEER was measured using an endothelial volt/ohm meter (TEER-EVOM2). Permeability of the endothelial layer was determined by measuring the passage of FITC fluorescently labeled dextran molecules through endothelial cell monolayers. The concentration of FITC-Dextran that diffused from the upper to the lower chamber was determined using a microplate reader, and intensity normalized to the control. Data are presented as the mean ± SD and were analyzed by one-way ANOVA. $^{**}p < 0.01$, $^{\#\#}p < 0.01$ compared to control and oTau treated group, respectively.

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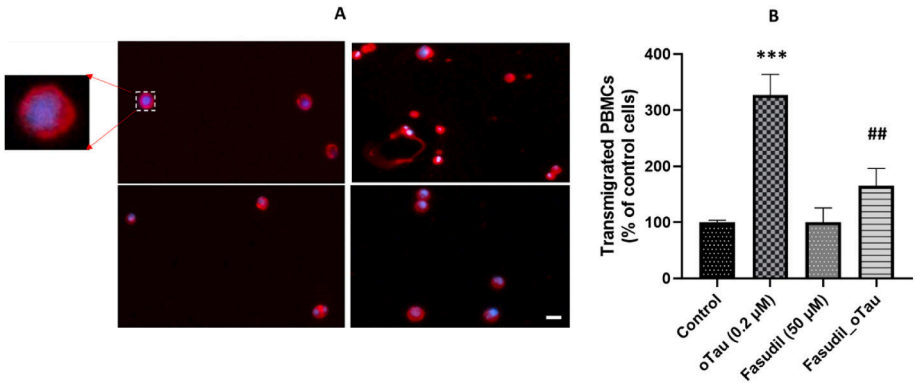

Fig. 6.

oTau increases PBMCs transmigration across the *in vitro* BBB model. (A) PBMCs that transmigrated from apical chamber to basolateral chamber of inserts were stained with APC conjugated anti mouse CD45 antibody and quantified using Nikon Eclipse Ti fluorescence microscope. (B) The percentage of transmigrated PBMCs was quantified and compared to the control. Data are presented as the mean ± SD of individual experiments, run in triplicate. At least 10 images were analyzed for each group (n = 10). ***$p < 0.001$ and $^{\#\#}p < 0.01$ compared with control and oTau treated group, respectively. Scale bar is 10 μM.

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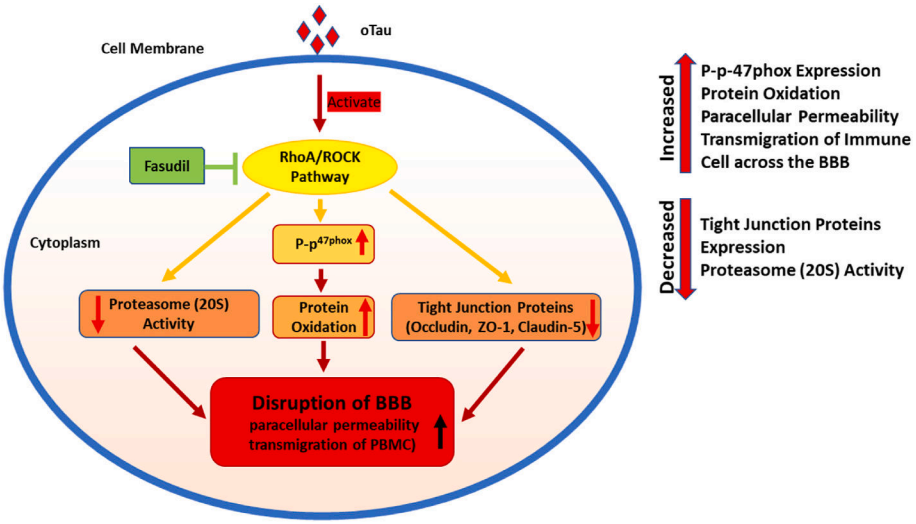

Fig. 7.

The Role of the RhoA/ROCK signaling pathway in oTau-induced disruption of BBB in CECs. oTau-induced activation of the RhoA/ROCK pathway can disrupt BBB and alter cerebral endothelial permeability. Fasudil (RhoA/ROCK signaling inhibitor) has neuroprotective effects on CECs. Regulation of the RhoA/ROCK pathway can be a potential therapeutic strategy to maintain the BBB integrity in AD.