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Inflammatory Cytokine IL-1β Downregulates Endothelial LRP1 via MicroRNA-mediated Gene Silencing

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Abstract

Effective clearance of neurotoxic amyloid-beta (Aβ) from the brain is a critical process to prevent Alzheimer's disease (AD). One major clearance mechanism is Aβ transcytosis mediated by low-density lipoprotein receptor-related protein 1 (LRP1) in capillary endothelial cells. A marked loss of endothelial LRP1 is found in AD brains and is believed to significantly impair Aβ clearance. Recently, we demonstrated that pro-inflammatory cytokines IL-1β, IL-6 and TNF-α, significantly down-regulated LRP1 in human primary microvascular endothelial cells (MVECs). In this study, we sought to determine the underlying molecular mechanism by which IL-1β led to LRP1 loss in MVECs. Reduced LRP1 protein and transcript were detected up to 24 hours post-exposure and returned to the baseline levels after 48 hours post-exposure with 1 ng/ml IL-1β. This reduction was in part mediated by microRNA-205–5p, −200b-3p, and −200c-3p, as these microRNAs were concomitantly upregulated in MVECs exposed to IL-1β. Synthetic microRNA-205–5p, −200b-3p, and −200c-3p mimics recapitulated LRP1 loss in MVECs without IL-1β, and their synthetic antagomirs effectively reversed IL-1β-mediated LRP1 loss. Importantly, we found that the expression of these three microRNAs was controlled by NF-κB as pharmacological NF-κB inhibitor, BMS-345541, inhibited the IL-1β-mediated upregulation of these microRNAs and rescued LRP1 expression. siRNA-mediated silencing of IκB in MVECs elevated microRNA-200b-3p and decreased LRP1 transcript, partially confirming our overall findings. In conclusion, our study provides a mechanism by which pro-inflammatory IL-1β instigates the suppression of LRP1 expression in MVECs. Our findings could implicate spatiotemporal loss of LRP1 and impairment of the LRP1-mediated clearance mechanism by endothelial cells.

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AUTHOR CONTRIBUTIONS

Heng-Wei Hsu and Masashi Kitazawa conceived the idea, designed the experiments, and wrote and revised this manuscript. Heng-Wei Hsu, Carlos J. Rodriguez-Ortiz and Joannee Zumkehr performed the experiments, interpreted the results and data analysis. Masashi Kitazawa performed supervision of overall research project, acquired resources and funding, and developed methodology. All authors approved the current version of the manuscript.

DISCLOSURE STATEMENT

The authors have no conflict of interest to report.

Keywords

Interleukin-1β; endothelial LRP1; microRNA; NF-κB; neuroinflammation; Alzheimer's disease

INTRODUCTION

Abnormal buildup of amyloid-beta (Aβ) in the brain is one of the pathological hallmarks of Alzheimer's disease (AD). Its accumulation precedes years and decades before the clinical onset of the disease and has long been predicted to be caused primarily by the overproduction of Aβ in the brain. However, in recent years, growing bodies of evidence from humans highlight that impaired clearance, rather than overproduction, of Aβ contributes to the pathological buildup of Aβ and increasing the risk for AD. The number of mechanisms in the brain including, but not limited to, phagocytosis and containment by microglia (Lee and Landreth, 2010), antibody-mediated clearance (Wilcock et al., 2003), enzymatic degradation by neprilysin (NEP) and insulin degrading enzyme (IDE) (Jha et al., 2015), clearance by interstitial fluid, cerebrospinal fluid (CSF) and glymphatic system (Tarasoff-Conway et al., 2015), and transcytosis through the blood-brain barrier (BBB) (Deane et al., 2009) are capable of clearing Aβ species. Among them, previous studies estimate that over 75% of Aβ clearance in transgenic mouse models of AD is mediated by transcytosis through the BBB (Shibata et al., 2000; Deane et al., 2003). Low-density lipoprotein receptor-related protein 1 (LRP1) and P-glycoprotein receptor are among putative receptors expressed in the capillary endothelial cells mediating transcytosis of Aβ (Storck et al., 2018). LRP1 is a ubiquitous receptor expressed not only in endothelial cells but also in astrocytes (Liu et al., 2017), neurons (Kanekiyo et al., 2013) and pericytes (Ma et al., 2018) in the brain. It is composed of a α-chain (515 kDa) and a β-chain (85 kDa) and capable of binding to multiple ligands including apolipoproteins, APP and Aβ (Shinohara et al., 2017). In mice, selective ablation of LRP1 in endothelial cells resulted in a significant accumulation of Aβ40 in the brain, leading to 2-fold elevated buildup of Aβ plaques (Storck et al., 2016). Endothelial LRP1 expression is found to be reduced with advancing age or exposure to environmental risk factor for AD (Silverberg et al., 2010; Singh et al., 2013; Kitazawa et al., 2016; Hsu et al., 2019), suggesting that faulty of LRP1-mediated Aβ clearance is one of feasible mechanisms by which aging and/or environmental risk factors accelerate the pathological progression of AD.

The underlying mechanisms of LRP1 loss in capillary endothelial cells and its pathological implication to AD remain to be investigated further. In chronic exposure to copper, one of putative environmental risk factors for AD, LRP1 appears to be reduced through proteosomal degradation (Kitazawa et al., 2016) and/or microRNA (Hsu et al., 2019). Recently, we also demonstrated that several pro-inflammatory cytokines including IL-1β, IL-6 and TNF-α mediate the downregulation of LRP1 in human primary microvascular endothelial cells (MVECs) (Kitazawa et al., 2016). Chronic inflammatory conditions are commonly observed in AD, and we hypothesize that such inflammatory changes in the brain microenvironment during the disease progression further promote a reduction of LRP1 in endothelial cells and contribute to the buildup of Aβ pathology. In this study, we investigated the underlying molecular mechanisms by which IL-1β reduced endothelial LRP1 via

microRNA-mediated silencing. We found three microRNAs, miR-205–5p, miR-200b-3p and miR-200c-3p, are particularly critical for LRP1 loss. Application of microRNA mimics alone sufficiently suppressed LRP1 and microRNA inhibitors recovered LRP1 expression, confirming these 3 microRNAs' role in silencing LRP1. We also observed that IκB kinase inhibitor BMS-345541 reversed IL-1β indecued-LRP1 downregulation, implying NF-κB signaling involved in this mechanism. Overall, our results revealed that pro-inflammatory cytokine IL-1β could increase NF-κB signaling and the expression of microRNAs to downregulate LRP1 in MVECs, thus, neuroinflammation may contribute to AD.

MATERIALS AND METHODS

Cell Culture and Treatments

Human primary brain microvascular endothelial cells (MVECs) were obtained from Cell Systems (#ACBRI 376) and maintained in complete classic media (#4Z0–500, Cell Systems) with subculture once every three days as described (Hsu et al., 2019). IL-1β at 1 ng/ml was added to the culture media for 24 hr-exposure (24h), then aspirated and replaced with fresh media for additional 24 hrs (24–24h) and 48 hrs (24–48h), respectively, in order to quantify the levels of LRP1 and microRNAs in MVECs. After identifying 3 microRNA candidates, MVECs were seeded onto 6-well plates at 50% confluency overnight, then 8 nM of hsa-miR-205–5p, 200b-3p, 200c-3p synthetic oligonucleotides or human negative control 2 scramble oligonucleotides (all from MISSION microRNA Mimics, Sigma-Aldrich) were transiently transfected to MVECs using RNAiMAX (#13778150, Thermo Fisher Scientific), in order to examine the direct effect of these microRNAs on LRP1 expression. We also transfected synthetic microRNA antagomirs to inhibit endogenous microRNA-205–5p, −200b-3p, and −200c-3p in MVECs using RNAiMAX, then 1 ng/ml IL-1β was added to the media. For siRNA treatment, MVECs were seeded onto 6-well plates at 50% confluence overnight, then 40 nM IκB siRNA reagent (#L-004765–00-0005, Dharmacon) or non-targeting control siRNA (#D-001810–10-05, Dharmacon) was added to the culture media. After 48 hrs of incubation with siRNA, cells were lysed by M-PER extraction buffer (#78501, Thermo Fisher Scientific) supplemented with 1:100 volume of proteases and phosphatases inhibitors (Sigma-Aldrich). Lysates were agitated by pipetting for 20 times and centrifuged at 10,000 g for 30 mins at 4°C. Supernatant was used to collect total protein or RNA as described below. All experiments were performed at least 3 times in duplicates or triplicates.

Quantitative Real Time-PCR

Following the extraction and purification of RNAs from MVECs by Direct-zol RNA MiniPrep kit (#R2052, Zymo Research), complementary DNA (cDNA) was synthesized by microRNA synthesis kit (#G903, Applied Biological Materials) or by iScript cDNA Synthesis kit (BioRad). One-tenth of diluted cDNA was amplified on a CFX Connect Thermocycler (BioRad) using SYBR Green Supermix (#1725121, BioRad) with the following conditions: for microRNAs, 95°C for 10 mins; 40 cycles (95°C for 15 secs, 60°C for 30 secs); dissociation curve 65°C - 95°C with 0.5°C increases every 5 secs, and for mRNAs, 95°C for 10 mins; 35 cycles (95°C for 30 secs, 60°C for 1 min, 72°C for 1 min); dissociation curve 60°C - 95°C with 0.5°C increases every 5 secs. Data were normalized to

internal control RNA U6 (microRNAs) or GAPDH (mRNAs). Primer sequences were selected via Primer3 software. A complete list of the primer sequences used in the present study is shown in Table 1.

Immunoblotting

Ten micrograms of protein were loaded and run on Mini-PROTEAN Precast Protein Gels (#4561085, BioRad), then transferred onto PVDF membranes. Membranes were blocked with Odyssey blocking buffer (#927–50003, Li-COR) at room temperature for 1 hr, further incubated with one or two of the primary antibodies overnight: LRP1 (#sc-57351, 1:25, Santa Cruz Biotechnology), NF-κB (#8242, 1:1000, Cell Signaling Technology), IκB (#9242, 1:1000, Cell Signaling Technology), GAPDH (#sc-25778, 1:5000, Santa Cruz Biotechnology) in TBS Odyssey blocking solution + 0.2% Tween20 at 4°C. After washing three times with 0.1% TBS-Tween20 buffer, membranes were incubated with the specific IRDye secondary antibodies (1:10000, LI-COR) for 1 hr in 5% non-fat milk/0.1% TBS-Tween20 buffer. Membrane blots then were scanned in an Odyssey infrared imager (LI-COR) and protein quantification was analyzed with Image Studio software (LI-COR). Internal control GAPDH, tubulin or histone-3 was used for protein normalization.

Electrophoretic Mobility Shift Assay (EMSA)

Nuclear proteins were extracted from MVECs using NE-PER™ nuclear and cytoplasmic extraction reagents (#78833, Thermo Fisher Scientific), and 5 μg of nuclear proteins were mixed with IRDye700 NF-κB Consensus Oligonucleotide (#829–07924, LI-COR) following manufacturer's Odyssey EMSA buffer Kit instructions (#829–07910, LI-COR). Raji nuclear extract was from Raji Burkitt's Lymphoma cell line (#36023, Active Motif) and used as a quality positive control for NF-κB activation. Reaction mixtures were wrapped with foil to avoid light and incubated at room temperature for 30 mins, then loaded and run on 5% TBE Gel (#4565015, BioRad). Blots were scanned in an Odyssey infrared imager (LI-COR) and protein quantification was analyzed with Image Studio software (LI-COR).

Statistical analysis

We analyzed the data for statistical significance using one-way analysis of variance (ANOVA) followed by Bonferroni's post hoc test for experiments with more than 2 groups, or using unpaired Student's t-test for experiments within 2 groups. All the data were performed at least three independent experiments and all figures were presented with the box-and-whisker plots. The box represents values of the median, the first (25^{th}) quartile and third (75^{th}) quartiles. The whisker shows the 5^{th} and 95^{th} percentile as well as all data points were shown on the graph. Results with $p < 0.05$ were considered statistically significant.

RESULTS

IL-1β decreases endothelial LRP1 transcript expression while upregulates microRNA-205–5p, 200b-3p, and 200c-3p

We previously demonstrated that IL-1β exposure decreased LRP1 protein levels in MVECs (Kitazawa et al., 2016). To extend our investigation and determine the underlying mechanisms by which IL-1β modulates LRP1 expression, we first sought to determine the

temporal recovery of LRP1 following the 24-hr exposure to IL-1β in MVECs. LRP1 protein as well as its transcript were significantly reduced immediately after 24-hr exposure (24h) and remained to be repressed even after additional 24-hr recovery without IL-1β (24h-24h, Fig. 1A, B). At 48 hrs of recovery (24h-48h), while the LRP1 protein levels remained reduced in MVECs exposed to IL-1β, the LRP1 transcript levels returned to control levels (Fig. 1A, B). In order to elucidate the underlying mechanism by which IL-1β reduced LRP1 expression in MVECs, we assessed the temporal changes in the three microRNAs (200b-3p, −200c-3p and −205–5p), which were identified from 7 putative microRNAs to silence LRP1 in MVECs following copper exposure (Hsu et al., 2019). These 3 microRNAs were significantly upregulated in MVECs immediately after 24-hr exposure to IL-1β (Fig. 2A) and after additional 24-hr recovery (Fig. 2B), and it finally returned to the baseline at 48-hr recovery (Fig. 2C). This temporal pattern was similar to that of LRP1 as described above.

MicroRNA-205–5p, −200b-3p, and −200c-3p mediated 1L-1β-induced downregulation of LRP1 in MVECs

We next examined if microRNA-205–5p, −200b-3p, and −200c-3p post-transcriptionally regulated LRP1 expression in MVECs, synthetic microRNA oligonucleotides (mimics) of microRNA-205–5p, −200b-3p or −200c-3p were transiently overexpressed, the expression and protein levels of LRP1 were quantified. After 48h-transfection with 8 nM of microRNA-205–5p, −200b-3p or −200c-3p mimics, MVECs exhibited over 50% reduction in LRP1 protein while a scrambled sequence of negative control oligonucleotides did not (Fig. 3A), suggesting that these 3 candidate microRNAs could downregulate LRP1 in MVECs. On the other hand, unlike IL-1β exposure (Fig. 1B), these microRNA mimics failed to reduce the expression of LRP1 transcript after 24-hr exposure (Fig. 3B).

Next, we sought to determine whether IL-1β-induced reduction of LRP1 was primarily mediated by microRNA-205–5p, −200b-3p, and −200c-3p in MVECs. We first transfected individual microRNA antagomir (up to 4 nM) to MVECs then exposed to IL-1β (1 ng/ml) for 24 hrs, but we failed to rescue LRP1 (data not shown). We then transfected all three antagomirs at concentration of 4 nM each and found a significant restoration of LRP1 protein levels and its transcript following the IL-1β exposure (Fig. 3C, D). Our findings showed that all three microRNAs might play a role and downregulate LRP1 during the IL-1β exposure as the application of three combined microRNA antagomirs, but not application of single microRNA antagomir, effectively rescued IL-1β-induced LRP1 downregulation in MVECs in a dose-dependent manner.

NF-κB signaling is involved in the microRNAs and LRP1 regulation after IL-1β exposure

The regulatory machinery of microRNA expression controlled by IL-1β stimulation in endothelial cells has not been investigated. Upon binding to its receptors, IL-1β activates NF-κB and AP-1 transcription factors through MyD88 and IRAK intracellular signaling pathways (Cui et al., 2010; Hein et al., 2010; Kitazawa et al., 2011; Hossen et al., 2017). Activation of these transcription factors is well defined to regulate inflammatory-related gene expression and has been shown to play a critical role in promoting AD pathology (Vukic et al., 2009; Cheng et al., 2013). We predicted that microRNA-205–5p, −200b-3p, and −200c-3p were also in part regulated by these transcription factors activated by IL-1β

signaling. Thus, we first searched predicted binding sites of NF-κB and AP-1 on putative promoter and regulatory regions of these three microRNAs using PROMO algorithm (http://alggen.lsi.upc.es/cgi-bin/promo_v3/promo/promoinit.cgi?dirDB=TF_8.3) and found that microRNA-205–5p, −200b-3p and −200c-3p all possessed 4, 5, and 3 putative NF-κB binding domains within 3 kb upstream of the coding region, respectively. In addition, it has been reported that NF-κB upregulates microRNA-205 expression in lung cancer cell lines (Cai et al., 2013). Therefore, we tested whether NF-kB signaling played a key role in modulating the expression of the three microRNAs in IL-1β-stimulated MVECs and contributed to the repression of LRP1. We inhibited NF-κB activation by applying BMS-345541, an inhibitor of IκB kinase (Burke et al., 2003), at concentrations of 5 or 10 μM with or without IL-1β (1 ng/ml) for 24 hr, then measured LRP1 protein levels in MVECs. BMS-345541 effectively reversed the IL-1β-mediated reduction of LRP1 in a concentration-dependent manner (Fig. 4A). We also observed that BMS-345541 reversed the reduction of LRP1 transcript (Fig 4B). Intriguingly, we found a significant reduction of the three microRNAs in MVEC received co-treatment with 10 μM BMS-345541 and 1 ng/ml IL-1β (Fig 4C). We performed electrophoretic mobility shift assay (EMSA) as well as western blot to confirm that 1 ng/ml IL-1β treatment activated nuclear NF-κB at 0.5 hr, while 10 μM BMS-345541 reversed the activation of NF-κB by IL-1β within 3h (Fig. 5A, B).

We next transiently knocked down IκB by siRNA to activate NF-κB in MVECs without IL-1β exposure and examined if it elicited the same effects as IL-1β did. Up to 47% reduction of IκB was achieved by the siRNA-mediated silencing, which resulted in about 4.7-fold increase in the nuclear translocation of p65 subunit at 48-hr post transfection in MVECs (Fig. 6A, B). The LRP1 protein and transcript levels were consequently and significantly decreased by ~60% and ~20%, respectively (Fig. 6C, D). On the other hand, siRNA-mediated silencing of IκB and subsequent activation of NF-κB were not sufficient to upregulate all the three microRNAs in MVECs. Only microRNA-200b-3p was found to be significantly upregulated to the similar levels with IL-1β exposure while microRNA-205–5p and −200c-3p remained unchanged (Fig. 6E). These findings showed that NF-κB activation was partly involved in the regulation of microRNAs and LRP1 in MVECs, but more complicated signaling cascades elicited by IL-1β controlled the full-spectrum of cellular responses described above.

DISCUSSION

Our previous study showed that pro-inflammatory cytokines, IL-1β, IL-6 and TNF-α mediate the downregulation of LRP1 in MVECs (Kitazawa et al., 2016). In this study, we further explored the potential underlying mechanism that microRNAs played a pivotal role to downregulate endothelial LRP1. In human primary MVECs, among 7 candidate microRNAs that were predicted to interact with the 3’UTR of LRP1 mRNA only microRNA-205–5p, −200b-3p, and −200c-3p upregulated after exposed to IL-1β. During the recovery phase, the restoration of LRP1 was inversely correlated with the temporal pattern of the reduction of these microRNAs. Meanwhile, pharmacological application of synthetic microRNA mimics and combined treatment of specific microRNA antagomirs with IL-1β further supported the direct involvement of modulating endothelial LRP1 by these

microRNAs. In addition, our results also indicate that microRNA-205–5p, −200b-3p and −200c-3p could be regulated by the NF-κB signaling in various degrees. However, our results also shows that only microRNA-200b-3p is significantly upregulated when IκB is knocked down in MVECs, suggesting that microRNA-200b-3p is the most responsive microRNA among the three microRNAs by NF-κB activation. While we do not have direct evidence, this may be in part due to the number of putative NF-κB binding domains in the promoter region. Five putative NF-κB binding domains were detected in the promoter region of microRNA-200b-3p, as opposed to 4 and 3 putative domains in microRNA205–5p and −200c-3p, respectively. Of course, many other transcription factors and/or cellular pathways are involved in the regulation of these microRNAs when MVECs are stimulated by IL-1β, and it remains premature to rule out other interactions of NF-κB with these microRNAs. Nonetheless, NF-κB-regulated microRNAs in the brain may contribute to neurodegenerative mechanisms as previously reported on microRNA-125b (Zhao et al., 2014), microRNA-146a (Cui et al., 2010) and microRNA-155 (Devier et al., 2015).

LRP1 plays an important role in brain Aβ clearance through endothelial cells (Storck et al., 2016), vascular muscle cells (Kanekiyo et al., 2012), astrocytes (Liu et al., 2017) and neurons (Kanekiyo et al., 2013). Accelerated loss of endothelial LRP1 is observed not only in advancing age but also in early stages of AD, suggesting that it is one of the initial pathological changes leading to AD (Kang et al., 2000). This is further supported by a recent study demonstrating that 5xFAD mice with selective ablation of endothelial LRP1 result in an elevated accumulation of Aβ40 in the brain and are sufficient to accelerate cognitive decline (Storck et al., 2016). These findings confirm that vascular endothelial cells mediate a major part of Aβ clearance in the brain (van Beijnum et al., 2008; Storck et al., 2016). Similarly, LRP1 also contributes to Aβ clearance via LRP1-mediated uptake and lysosomal degradation in neurons (Kanekiyo et al., 2013) and via upregulation of matrix metalloproteinases (MMPs) and various Aβ-degrading enzymes including NEP, IDE, angiotensin converting enzyme and plasmin (Dries et al., 2012; Etique et al., 2013). LRP1 in astrocytes also regulates the expression of IDE, MMP2 and MMP9, and contributes to Aβ degradation (Mulder et al., 2012; Liu et al., 2017). On the contrary, LRP1 in vascular smooth muscle cells may negatively impact on Aβ degradation as its down-regulation is reported to activate NEP and IDE to prevent the vascular Aβ accumulation and cerebral amyloid angiopathy among AD patients (Miners et al., 2006; Kanekiyo et al., 2012). Interestingly, LPR1 in neurons is also suggested to be involved in pathological tau propagation in a mouse model (Rauch et al., 2020). Collectively, these findings, whether neuroprotective or detrimental, validate a critical role of LRP1 in AD, and its role could be dependent on cell types. Thus, further investigations are urged to better elucidate the precise contribution of LRP1 in different cell types to the pathogenesis of AD.

NF-κB is a ubiquitous nuclear transcription factor and is well known to play a critical role not only in inflammation but also in neuronal plasticity, learning and memory functions, hence its dysregulation can be tightly associated with the progression AD phenotypes (Mattson and Camandola, 2001). In post-mortem AD brains, NF-κB is particularly activated in neurons and astrocytes around Aβ plaques (Kaltschmidt et al., 1997; Lukiw and Bazan, 1998), which may result in accelerated neuronal loss or aberrant neuroinflammatory activation as evidenced by elevated levels of pro-inflammatory cytokines including IL-1β in

the brain, CSF, and plasma of AD patients (Licastro et al., 2000; Mishra et al., 2012). It is also well documented that IL-1β and many other pro-inflammatory cytokines activate NF-κB signaling and could serve as a vicious feedback activation of inflammatory activation in AD (Xie et al., 2015). Recent studies report that loss of target of Myb1, a negative regulator of the interleukin-1β receptor-1, could increase Aβ depositions (Martini et al., 2019) and by blocking IL-1β signaling attenuates Aβ and Tau pathology in 3xTg-AD mice (Kitazawa et al., 2011). These results confirm the importance of IL-1β signaling in neuroinflammation and AD. Nevertheless, other studies report potentially contradicting results that overexpression of IL-1β reduces Aβ-related pathology by promoting non-amyloidogenic APP cleavage, enhancing microglial phagocytic capacity and preventing neuronal apoptosis, suggesting that IL-1β exhibits a neuroprotective role in mouse models (Shaftel et al., 2007; Tachida et al., 2008; Matousek et al., 2012). Thus, IL-1β can be neuroprotective or detrimental depending on its bioavailability, duration, timing, type of cells to interact, and other factors associated with its physiological and pathological roles.

Our result is the first to show that IL-1β exposure particularly induces the expression of microRNA-205–5p, −200b-3p, and −200c-3p and further downregulates endothelial LRP1. Although the pathological involvement of microRNAs in AD remains largely undetermined, growing evidence highlights that the spatiotemporal dysregulation of microRNA expression and maturation could easily perturb neuroprotective or physiological functions of various cells, which may contribute critically to development of AD pathology and cognitive decline. Aside from our findings, elevated levels of microRNA-200b represses beta-1, 4-mannosyl-glycoprotein 4-beta-N-acetylglucosaminyltransferase, an enzyme critical for Aβ phagocytosis (Fiala et al., 2007; Guedes et al., 2015). The number of microRNAs (microRNA-132–3p, −129–5p, −27a-3p, −92b-3p, −129–2-3p, −136–5p, −200a-3p, −370, −409–5p and −487a) are reported to be dysregulated in AD post-mortem brain samples, and these microRNAs are predicted to interact with *APP*, *BACE1*, *APOE*, and *MAPT* transcripts (Lau et al., 2013; Putteeraj et al., 2017; Jaber et al., 2019). In addition, microRNA is a suitable diagnostic biomarker in the CSF since it stays stable and presents several hundred-fold greater than Aβ peptides (Alexandrov et al., 2012). Recent studies also reported that anti-microRNA therapy could reduce neuroinflammation in stroke and traumatic brain injury mouse models (Henry et al., 2019; Talebi et al., 2019). Therefore, microRNA dysregulation plays a critical role in the inflammatory response and AD formation. Nevertheless, the effect of IL-1β on different cell types might trigger the activation of different microRNAs and contribute differently to the intricate inflammation mechanism. More investigation is needed to clarify whether these three novel microRNAs are endothelial cell-specific responses and whether they are highly upregulated in cerebrovascular endothelial cells in the brain from AD patients and mouse models of AD.

In conclusion, we unveil an important mechanism that pro-inflammatory cytokine IL-1β represses LRP1 expression through upregulation of microRNA-205-p, −200b-3p and −200c-3p in MVECs. Induction of these microRNAs is mediated by NF-κB activation, and pharmacological inhibition of NF-κB effectively reverses IL-1β-mediated loss of LRP1 in MVECs. Since endothelial LRP1 mediates Aβ clearance in the brain, chronic neuroinflammation may lead to parenchymal Aβ buildup and potentially contribute to the

onset of AD. It is worth of the investigation if restoration of endothelial LRP1 significantly delays the progression of AD neuropathology.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGMENT

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Abbreviations:

Aβ	amyloid-beta
AD	Alzheimer's disease
BBB	blood-brain barrier
CSF	cerebrospinal fluid
IDE	insulin degrading enzyme
IκB	NF-κB inhibitor
IKK	IκB kinase
IL-1β	interleukin-1β
IL-6	interleukin-6
LRP1	low-density lipoprotein receptor-related protein 1
MMP	matrix metalloproteinase
MVEC	human primary microvascular endothelial cell
NEP	neprilysin
NF-κB	nuclear factor-κB
TNF-α	tumor necrosis factor-α

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Highlights

1. IL-1β reduces LRP1 expression in microvascular endothelial cells.
2. Upregulation of microRNA-205–5p, −200b-3p and −200c-3p is responsible for IL-1β-mediated loss of LRP1.
3. NF-κB activation controls expression of these microRNAs and LRP1 in microvascular endothelial cells when exposed to IL-1β.

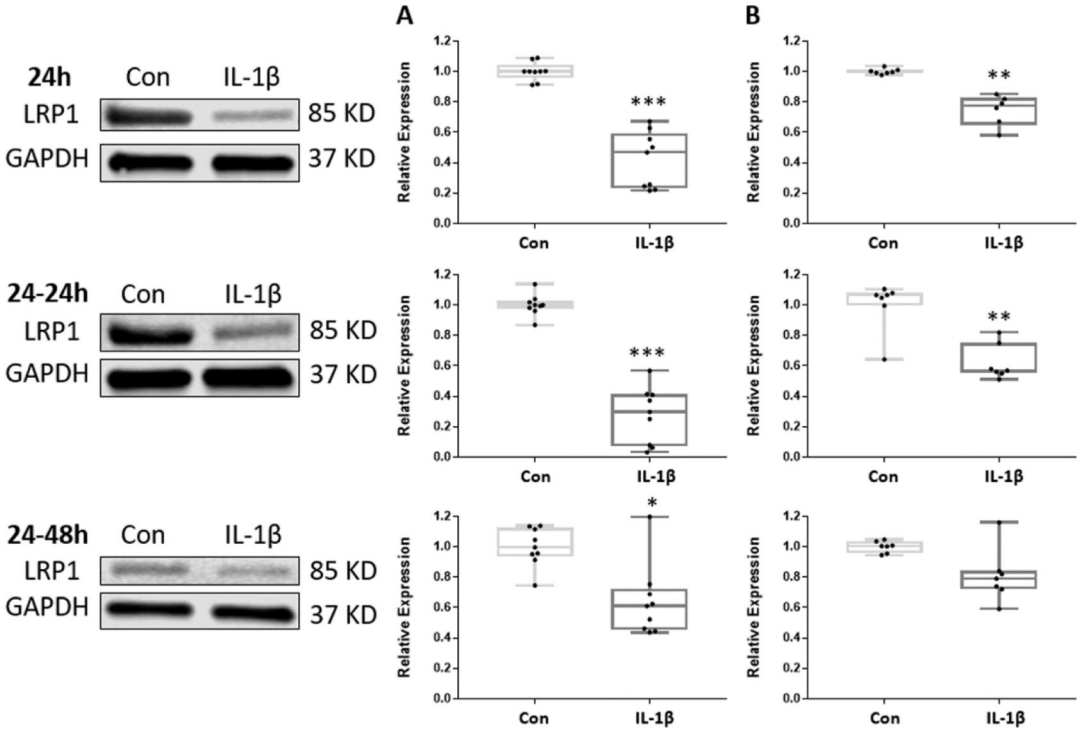

Figure 1. LRP1 is significantly reduced in MVECs exposed to IL-1β.

The temporal changes of **(A)** LRP1 protein levels and **(B)** the transcript of LRP1 are determined by immunoblotting and qRT-PCR, respectively. IL-1β-mediated loss of LRP1 in MVECs is significant immediately after 24 hrs exposure (24h), at 24-hr recovery (24–24h) as well as at 48-hr recovery (24–48h). On the other hand, LRP1 transcript returns to control level at 48-hr recovery post IL-1β exposure. The center line represents the median, the bottom and top lines of the box indicate the first and third quartiles, respectively. The tails show the 5^{th} - 95^{th} percentile values. All data were collected from three separate experiments in duplicates or triplicates, N=6–9 (*$p < 0.05$, **$p < 0.01$, ***$p< 0.001$ compared to control group). Relative expression of LRP1 is normalized to GAPDH.

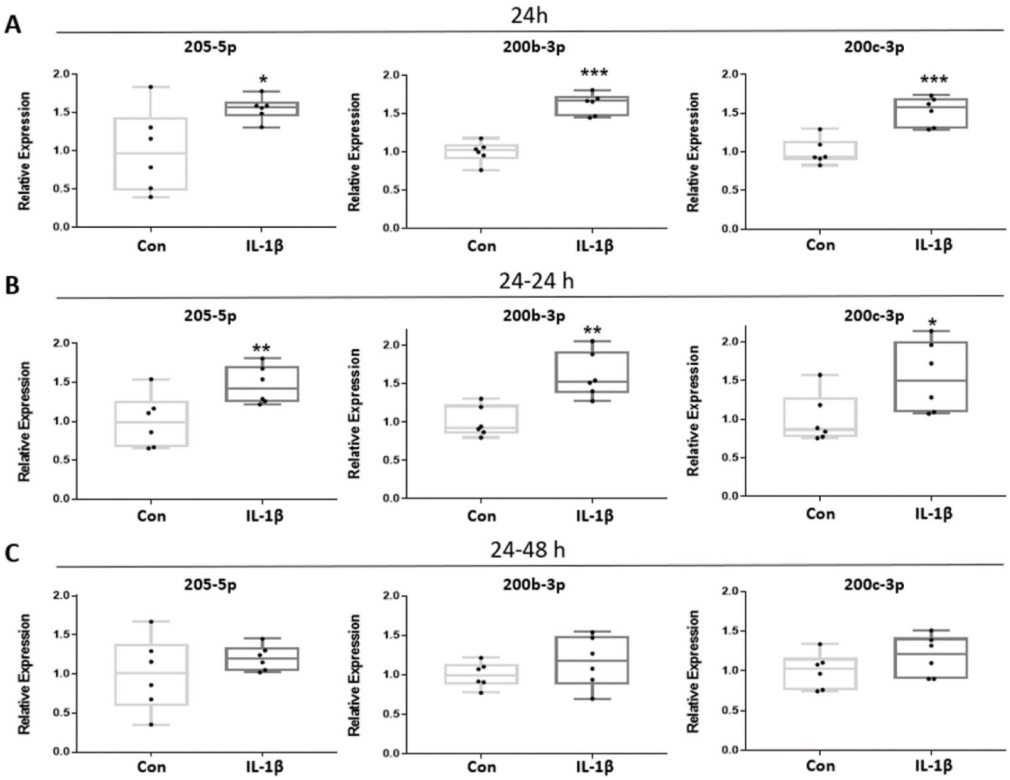

Figure 2. MicroRNA-205–5p, −200b-3p and −200c-3p are significantly elevated in MVECs when exposed to IL-1β.

Temporal changes of microRNA-205–5p, −200b-3p, and −200c-3p in MVECs are quantitatively measured at **(A)** immediately after the 24-hr exposure to 1ng/ml IL-1β (24h), **(B)** 24-hr recovery after IL-1β exposure (24–24h), and **(C)** 48-hr recovery after IL-1β exposure (24–48h) by qRT-PCR. These three microRNAs significantly increase immediately after IL-1β exposure, remain elevated at 24-hr recovery and return to the control levels at 48-hr recovery. The center line represents the median, the bottom and top lines of the box indicate the first and third quartiles, respectively. The tails show the 5^{th} - 95^{th} percentile values. All data were collected from three separate experiments in duplicates, N=6 (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ compared to control group). The microRNA expression is normalized to small nuclear RNA U6.

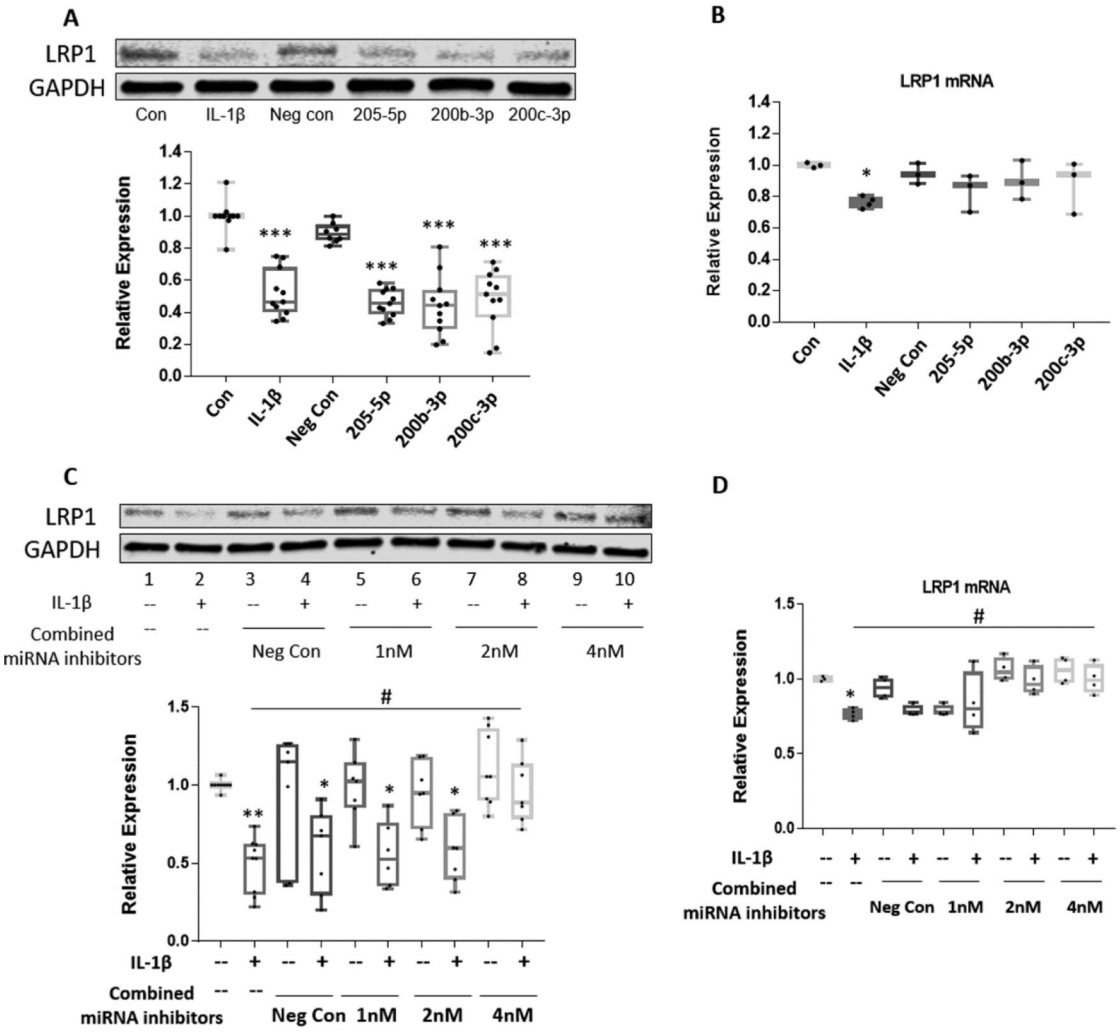

Figure 3. MicroRNA-205–5p, −200b-3p and −200c-3p mediate IL-1β-induced LRP1 loss in MVECs.

(A) Transfection of synthetic microRNA-205–5p, −200b-3p, and −200c-3p mimics (8 nM) significantly decreases LRP1 protein levels in MVECs. The data were collected from four separate experiments in at least duplicates, N=11 (***$p < 0.001$ compared to control group). **(B)** On the other hand, microRNA mimics do not decrease LRP1 mRNA transcript. The data were collected from three separate experiments, N=3 (*$p < 0.05$ compared to control group). **(C)** The combination of synthetic microRNA-205–5p, −200b-3p, and −200c-3p antagomirs (4 nM each) restores LRP1 protein levels following the exposure to IL-1β in MVECs. The data were collected from three separate experiments in triplicates, N=9 (*$p < 0.05$ and **$p < 0.01$ compared to its corresponding no IL-1β treatment group, and #$p < 0.05$ compared between IL-1β treatment group #2 and IL-1β + 4 nM antagomirs group #10). **(D)** The effect of the 3 combined antagomirs on LRP1 mRNA is assessed. The data were collected from four separate experiments, N=4 (*$p < 0.05$ compared to its corresponding no IL-1β treatment group, and #$p < 0.05$ compared between IL-1β treatment group and IL-1β + 4 nM antagomirs group). All data center line represents the median, the bottom and top lines of the box indicate the first and third quartiles, respectively. The tails show the 5^{th} - 95^{th} percentile

values. The results strongly support the direct post-transcriptional suppression of LRP1 by microRNA-205–5p, −200b-3p, and −200c-3p.

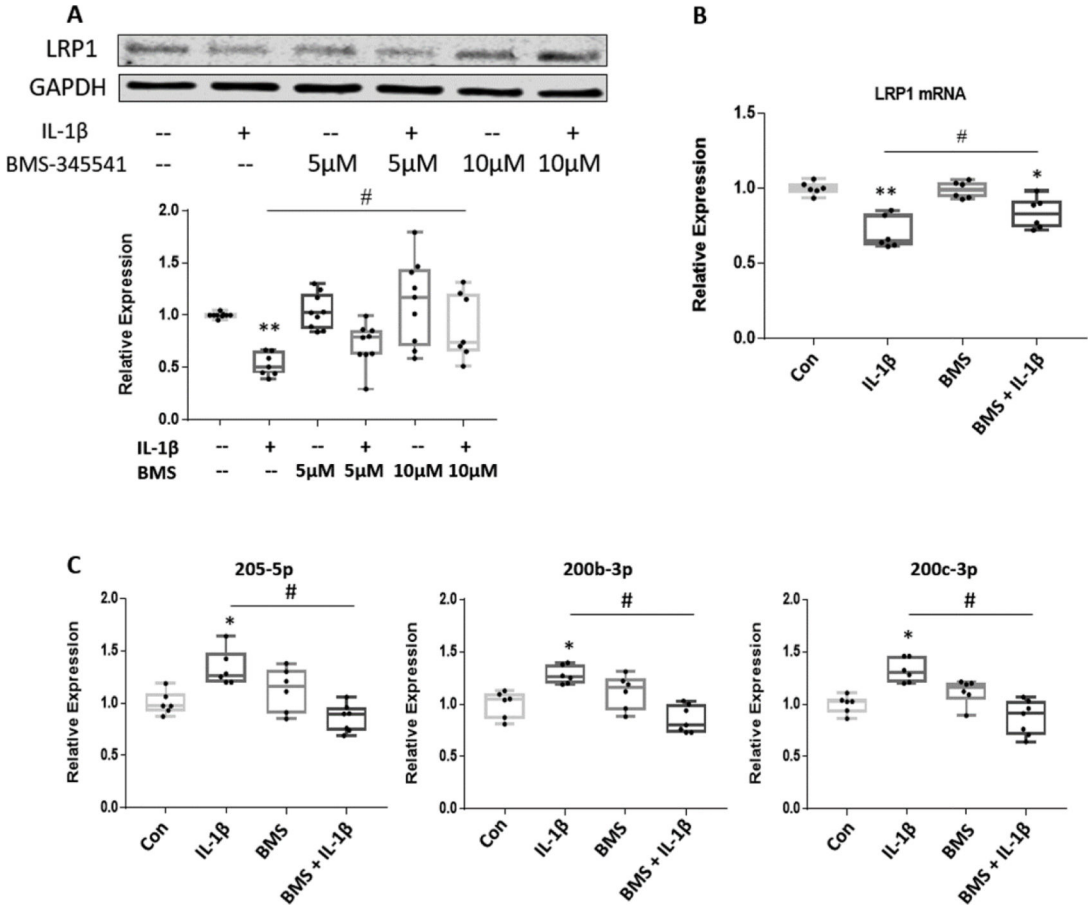

Figure 4. Inhibition of NF-κB prevents IL-1β-induced loss of LRP1 and upregulation of microRNA-205–5p, −200b3p, and −200c-3p.

(A) A pharmacological IκB inhibitor (BMS-345541, 10 μM) reverses the IL-1β-mediated loss of LRP1 in MVECs. Band intensity is normalized to GAPDH. The data were collected from three separate experiments in triplicates, N=9 (**p < 0.01 compared to control group, and #p < 0.05 compared between IL-1β treatment group and IL-1β + 10 μM BMS-345541 group). **(B)** BMS-345541 also attenuates IL-1β-mediated loss of LRP1 transcript. The data were collected from three separate experiments in duplicates, N=6 (*p < 0.05, **p < 0.01 compared to control group, and #p < 0.05 compared between IL-1β treatment group and IL-1β + 10 μM BMS-345541 group). **(C)** BMS-345541 treatment effectively suppresses IL-1β-mediated upregulation of microRNA-205–5p, −200b-3p and −200c-3p in MVECs. The microRNA expression is normalized to small nuclear RNA U6. The data were collected from three separate experiments in triplicates, N=9 (*p < 0.05 compared to control group, and #p < 0.05 compared between IL-1β treatment group and IL-1β + 10 μM BMS-345541 group). All data center line represents the median, the bottom and top lines of the box indicate the first and third quartiles, respectively. The tails show the 5^{th} - 95^{th} percentile values.

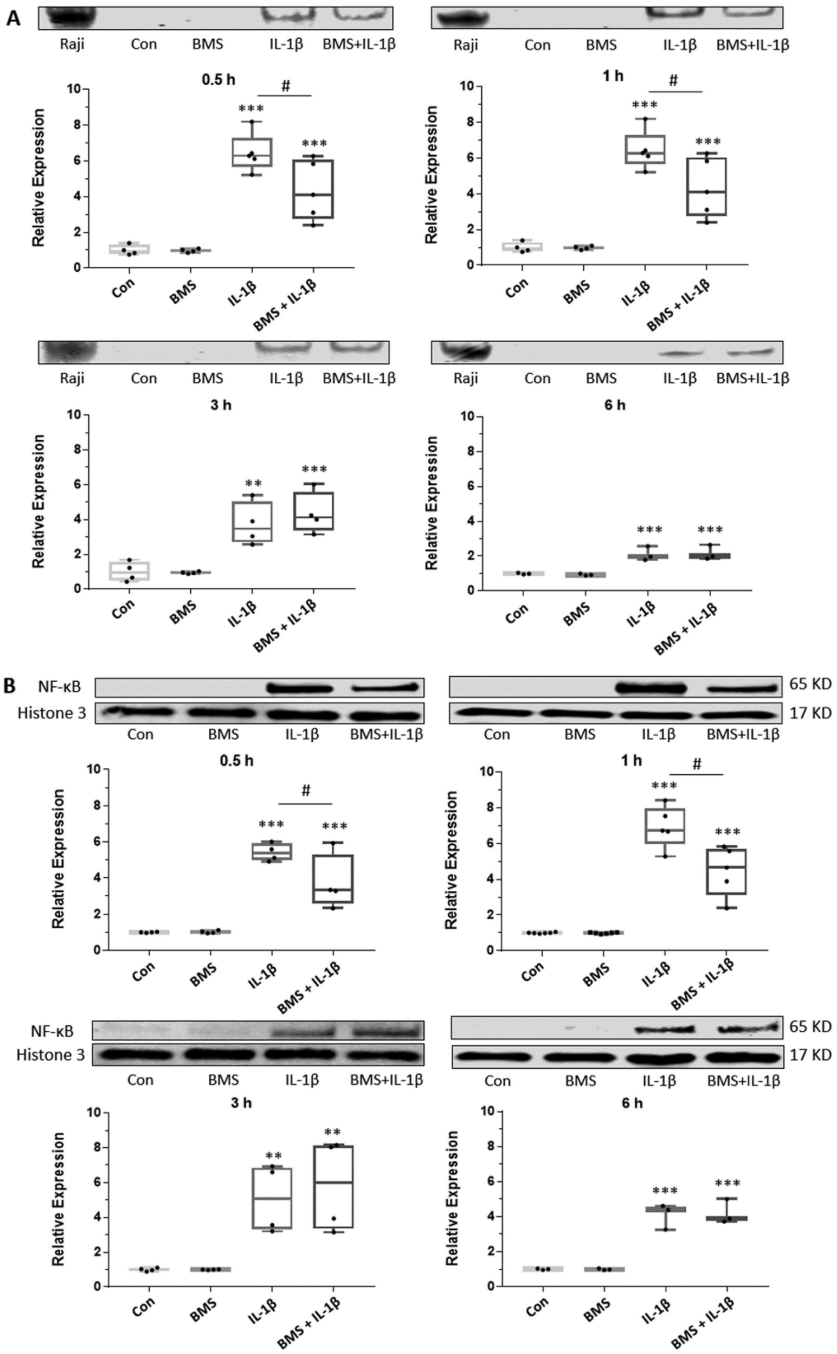

Figure 5. NF-κB is activated by IL-1β and is effectively inhibited by BMS-345541 in MVECs.

Nuclear proteins are extracted from MVECs treated with 1 ng/ml IL-1β with or without BMS-345541 at 0.5, 1, 3 and 6 hrs time points. (A) Nuclear NF-κB activity is determined by EMSA. Raji nuclear extract from Raji Burkitt's Lymphoma cells was used as a positive control. The data were collected from three to five separate experiments, N=3–5 (**$p < 0.01$ and ***$p < 0.001$ compared to control group, and #$p < 0.05$ compared between IL-1β treatment group and IL-1β + 10 μM BMS-345541 group). **(B)** Nuclear translocation of p65 subunit of NF-κB is determined by immunoblotting. The data were collected from three to

five separate experiments, N=3–5 (**p < 0.01 and ***p < 0.001 compared to control group, and #p < 0.05 compared between IL-1β treatment group and IL-1β + 10 μM BMS-345541 group). All data center line represents the median, the bottom and top lines of the box indicate the first and third quartiles, respectively. The tails show the 5^{th} - 95^{th} percentile values.

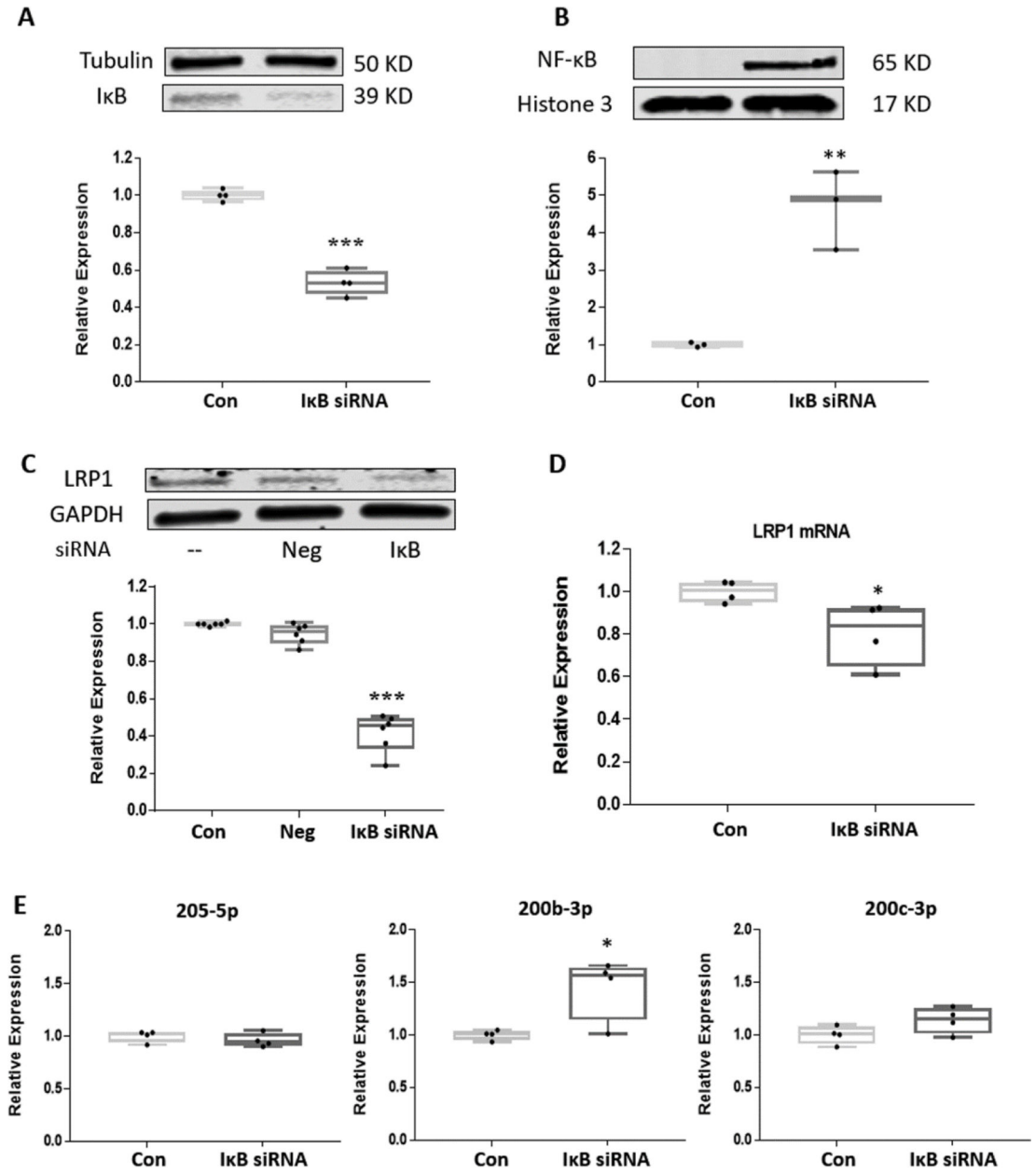

Figure 6. siRNA-mediated silencing of IκB in MVECs downregulates LRP1 and elevates microRNA-200b-3p, but not microRNA-205–5p or −200c-3p.

MVECs are transiently transfected with IκB siRNA (40 nM) for 48 hrs. **(A)** IκB is effectively knocked down by nearly 50% after successful transient transfection. The data were collected from two separate experiments in duplicates, N=4 (***$p < 0.001$ compared to control group). **(B)** Transient loss of IκB promotes nuclear translocation of p65 subunit of NF-κB. The data were collected from three separate experiments, N=3 (**$p < 0.01$ compared to control group). **(C)** Transient loss of IκB in MVECs significantly decreases LRP1 protein levels. The data were collected from three separate experiments in duplicates, N=6 (***$p < 0.001$ compared to control group). Negative control (Neg) indicates a transient transfection with scramble siRNA in MVECs. **(D)** Transient loss of IκB reduces the

expression of LRP1 transcript. The data were collected from four separate experiments, N=4 (*p < 0.05 compared to control group). **(E)** MicroRNA-200b-3p expression, but not microRNA-205–5p or −200c-3p, is significantly increased after the IκB knockdown in MVECs. The data were collected from four separate experiments, N=4 (*p < 0.05 compared to control group). All data center line represents the median, the bottom and top lines of the box indicate the first and third quartiles, respectively. The tails show the 5^{th} - 95^{th} percentile values.

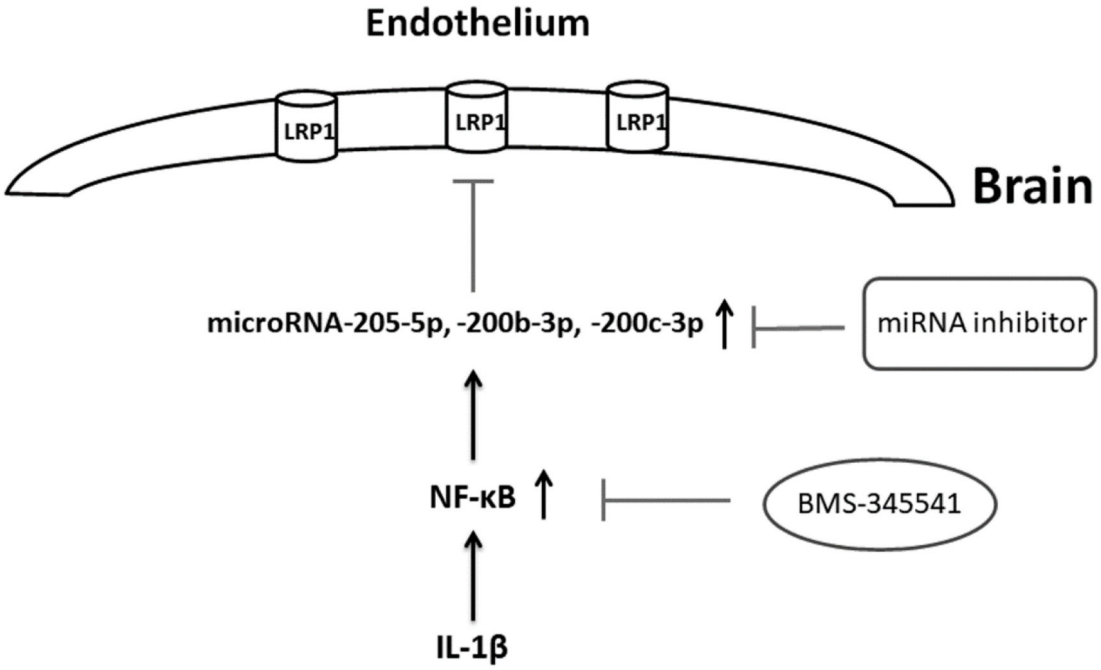

Figure 7. Putative mechanism of the IL-1β-mediated loss of endothelial LRP1.

We find IL-1β exposure activates NF-κB and upregulates three microRNAs (microRNA-205–5p, −200b-3p and −200c-3p), leading to a significant downregulation of LRP1 in endothelial cells. Our results also indicate a possible direct effect of NF-κB activation on the expression of LRP1 transcripts, which could be independent from microRNA-mediated LRP1 regulation. Effective and direct inhibition of NF-κB signaling and/or these microRNAs can attenuate potentially adverse effects of IL-1β on LRP1 in endothelial cells.

Table 1.

List of primer sequences used in the present study

Primer	Sequence
miR-103a-3p	5’- AGCAGCATTGTACAGGGCTATGA -3’
miR-107	5’- AGCAGCATTGTACAGGGCTATCA -3’
miR-200b-3p	5’- TAATACTGCCTGGTAATGATGA -3’
miR-200c-3p	5’- TAATACTGCCGGGTAATGATGGA -3’
miR-205–5p	5’- TCCTTCATTCCACCGGAGTCTG -3’
miR-214–5p	5’- TGCCTGTCTACACTTGCTGTGC -3’
miR-429	5’- TAATACTGTCTGGTAAAACCGT -3’
RNU6	5’- AAATTCGTGAAGCGTTCCAT -3’
LRP1 forward	5’- CAACGGCATCTCAGTGGACTAC -3’
LRP1 reverse	5’- TGTTGCTGGACAGAACCACCTC -3’
GAPDH forward	5’- GTCTCCTCTGACTTCAACAGCG -3’
GAPDH reverse	5’- ACCACCCTGTTGCTGTAGCCAA -3’